PROBLEMS *in* AMOEBIASIS

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PREFACE

THIS TREATISE was written in the hope that it might be useful to other investigators of problems in amoebiasis, since data available at the present time do not warrant many statements currently made concerning the growth requirements of *Entamoeba histolytica*, factors of its pathogenicity, its relationship to bacteria and/or other microorganisms in the intestinal flora, its metabolism, or concerning chemotherapy Although in problems of enteritis the importance of studies on *E. histolytica* can in many cases hardly be overemphasized such studies have not received attention of many prominent biochemists and physiologists, probably because the parasite has never been cultivated by itself, and the complications inherent in the activities of associated microorganisms or metazoan cells are formidable As pointed out by Stephenson* in her excellent book on bacterial metabolism studies on the nutrition of "difficult" pathogens are complicated by the complex and peculiar media, "that are rendered necessary owing to the inability of many parasitic organisms to synthesize for themselves certain molecules essential for growth" However, these difficulties have not proved insurmountable in cases where the pathogens have been obtainable *in vitro* in pure culture In the case of *E histolytica* all attempts to find adequate combinations of essential molecules, in the absence of other living things, have proved unsuccessful It appears, therefore, that a treatise on methods of approach is in order at the present time

In view of the complex interrelationships between amoebae and species of bacteria in the intestinal tract and in the test tube, a working knowledge of bacterial metabolism is a prerequisite for progress in problems of amoebiasis Such knowledge is obtainable only by those having had considerable training in

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*Stephenson M S *Bacterial Metabolism*, 3rd Edition London, Longmans, Green, 1949, 398 pp

organic chemistry and physics In addition, investigators of any disease, whether in man or other animals, must have training in clinical and pathological fields, or collaboration with clinicians and pathologists

In the absence of experimental data by which certain observations on cases of amoebiasis might be explained, investigators have had recourse to hypotheses and theories However, a number of theories on the relationships of *E. histolytica* to the host have furnished incentives for much diligent work which justifies advancement of the theories even though it may lead to their eventual abandonment In her discussion of this method of approach to problems, Stephenson has translated a statement by Pasteur with approval, to the effect that adoption of ideas that cannot be rigorously proved may be the best way of looking at things She was discussing the controversies on the ideas of the organic chemists, Berzelius, Wohler, and Liebig, and of Pasteur, concerning factors of fermentation, and investigations which were forerunners of our present-day concepts of the causation of disease. However, when by abuse of this method the investigator places greater emphasis on proving his theory than in evaluating all of the evidence *pro* and *con* he becomes partner in a situation wherein progress may be arrested rather than advanced As pointed out later in the present treatise there appear to have been illustrations of such abuse in the history of amoebiasis

The writer wishes to express appreciation for suggestions offered by fellow workers, particularly to Drs Victor H. Haas and Willard H Wright, Director and Assistant Director of the National Microbiological Institute, National Institutes of Health Drs Harry S Eagle, Theodor von Brand, John E. Tobie, Clarence A Imboden, Jr, Edmund J. Talbott, Harry D. Baernstein, Joseph Greenberg, Mr John Bozicevich, Miss D. Jane Taylor, and Mrs Ida L Bartgis have read and criticized all or parts of the manuscript. My associates Miss Lucy V. Reardon and Dr. Leon Jacobs have also read and criticized the manuscript and rendered invaluable service in preparing the indices and references to

literature Dr. Elizabeth Verder of the Laboratory of Infectious Diseases, National Microbiological Institute, Dr. Floyd S. Daft, Director, National Institute of Arthritis and Metabolic Diseases, National Institutes of Health, and Dr. Leslie Hellerman of the School of Medicine of Johns Hopkins University have also contributed valuable information and suggestions. Under the direction of Miss Inez Demonet of the Medical Arts Section of the National Institutes of Health, Mrs Frances Rose prepared drawings, and the Photographic Section under the direction of Mr Roy Perry prepared the photographs Dr Clarence A Imboden, Jr, now at the United States Hospital of Public Health at Carville, Louisiana, with whom the writer collaborated at Bethesda in researches on experimental amoebiasis, has kindly contributed Chapter 7, which is of especial interest to physicians.

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PROBLEMS IN AMOEBIASIS

INTRODUCTION

REGARDLESS of discrepancies among viewpoints of investigators at the present time concerning the host-parasite relationships in amoebiasis, whether *Entamoeba histolytica* invariably produces lesions, whether it may occasionally live within the lumen of the intestine as a commensal whether symbiosis of amoebae and bacteria is required for pathogenicity or whether in some cases the occurrence of *E histolytica* may have nothing to do with the patient's symptoms, there is general agreement that the majority of infected individuals are "carriers" They do not have symptoms that are demonstrable by methods usually employed On the other hand, amoebic dysentery and or manifestations of extraintestinal amoebiasis, such as abscess of the liver, lungs or other organs, are not of infrequent occurrence The disease is one of the most serious of those caused by protozoan parasites, and its distribution is cosmopolitan.

A Brief Résumé of Investigations on Amoebiasis

In a preliminary analysis of the history of observations and experiments on amoebiasis the writer felt that a clearer discussion might be provided by dividing the period from Loesch (1) 1875, to the present into three epochs based on what appeared to be the dominant opinion of each epoch In the first epoch extending from 1875 to about 1913 there were grave doubts among investigators that amoebae may cause dysentery Many of these doubts were removed by the outstanding experiments of Walker (2), and Walker and Sellards (3), on human volunteers Since some of these volunteers developed dysentery, with amoebae in their stools, following ingestion of material from cases of amoebiasis it appeared that the etiological relationship between *E histolytica* and dysentery was established In 1919 the situation was further

intestinal mucosa must always occur. French investigators, Brumpt (21), Deschiens (22, 23), and others, have advanced a theory that there are two species having the cytological characteristics of *E. histolytica* except as to size, one being nonpathogenic and the other pathogenic. A considerable number of investigators, including Meleney and Frye (24), and Anderson *et al.* (18), have produced and cited evidence for the occurrence of strains of *E. histolytica* differing in virulence. In discussing pathogenicity, Anderson and associates concede that many factors such as changes in the physiological state of the parasite and of the host may be operative. In this connection, the studies of Alexander and Meleney (25) are of particular interest. One community in Tennessee had a high incidence of infection with *E histolytica* with little evidence of pathogenicity. Another community having a high incidence had many cases of dysentery. Persons in the community with little evidence of pathogenicity had better diets, with a greater variety of foods, with richer vitamin content than those in the community with comparatively many cases of dysentery. There was greater use of milk and other foods containing vitamin D in the first community than in the second

A most attractive theory involves the participation (symbiosis) of the amoeba with certain species of bacteria in the development of pathogenicity. This theory has many advocates dating back even to the time of Loesch, and has been emphasized by other prominent students of the problem such as Councilman and Lafleur (26), Walker and Sellards (3), Westphal (27), and Cleveland and Sanders (28). Westphal infected himself with *E. histolytica* by swallowing material from a convalescent case of amoebic dysentery, becoming positive for the amoeba within several days but developing no symptoms of dysentery during a subsequent period of eight months. He then drank fluid from the stool of another case of amoebic dysentery from which the amoebae had been removed by passage through filter paper. Probably attributable to the bacteria occurring in this fluid he developed amoebic dysentery after twenty-three days. A friend without infection with *E. histolytica* also drank some of the

fluid but did not have dysentery Such evidence does not indicate that *E histolytica* is an obligate pathogen There appears no question, however, that *E histolytica* differs from the nonpathogenic species of intestinal amoebae

In a recent paper supporting the arguments of Reichenow, with some original observations, Hoare (19) showed that *E histolytica* ingests bacteria both *in vitro* and *in vivo* He postulated two phases in the life of trophic amoeba, in one of which it lives as a commensal and in the other as a pathogen According to this hypothesis the commensal amoebae are minute compared with those that are pathogenic, the cysts being derived from the lumen-dwelling organisms In carriers there is, according to the same hypothesis, no pathogenic phase However, although there is much evidence for the occurrence of at least two races of *E histolytica*, one race having small trophozoites and cysts compared with the other, it would be interesting to learn on what basis the occurrence of large and small trophozoites within the same race was ascertained by Reichenow, Hoare, and others Within the tissues, the lumen of the bowel, or even in the test tube the size of trophic amoebae is affected by nutritional factors In our laboratory for example, the trophic amoebae of a given race seen in cultures with certain single species of bacteria are much larger than those grown with other species or with *Trypanosoma cruzi* Size alone appears rather a poor criterion of pathogenicity or its absence

Since so little information is available concerning the inherent pathogenicity of *E histolytica*, its methods of attack, or the participation with the amoeba of other microorganisms in the production of clinical amoebiasis it is small wonder that only limited progress has been made in chemotherapy Chemotherapy is discussed in the final chapter of this treatise but as pointed out by Armstrong *et al* (37, 38, 39), and by Plichet (40) no regimen of treatment has been followed with success in eliminating *E histolytica* from all of the patients

Some Practical Problems in Amoebiasis

Probably attributable to progress during the past 50 years in methods of filtration and chemical treatment of water supplies, installment of toilet facilities in homes and offices, provision for drinking fountains, improvements in processing and packaging of foods, elimination of infected individuals from places where food is prepared and served to the public, elimination of unsanitary practices and vigilance in inspecting such places including food markets, development and enforcement of regulations concerning plumbing, screening of buildings against entry of insects, and general education of the public in matters of sanitation, the incidence of amoebiasis in countries where these provisions are carried out, with the exception of certain institutions, notably those for the feeble-minded or insane, is certainly much lower at the present time than in earlier periods Amberg (29) in 1901 noted the common occurrence of amoebic dysentery, as revealed by the literature, and described six cases of the disease in Baltimore, U.S A. in young children One "drank from the gutters" and the others lived in unsanitary surroundings.

Comprehensive data on the relationship of amoebiasis to living conditions in other countries were reported in a book by Blanc and Siguier (30) and other data by Chiray and Chene (31). However, in view of the paucity of comparative figures on the incidence of amoebiasis from the early years of the present century when effective methods of diagnosis were made available to the present time, some statistics on another fecal-borne disease, typhoid fever, have been obtained through the courtesy of Dr C. F Drake of the water filtration plant at Pittsburgh, Pennsylvania, and Drs. T. McC Mahon, and G. R. Lacy of the Medical School of the University of that city. These data are illustrated in Figure 1 which shows that the high incidence of this disease in 1908 when effective filtration of water was instituted has dropped to almost complete disappearance at the present time. As was pointed out by Dr. Lacy (32) in a discussion of these statistics, other factors such as those mentioned above in addition to filtration of water have contributed toward

the conquest of the disease Although no figures on amoebiasis in Pittsburgh could be obtained by the writer there has undoubtedly been a drop in the incidence of this disease. However, from data reported in later paragraphs it is probable that many cases could still be found on the instigation of effective studies In many other cities of the United States there is deplorable neglect of sewage treatment, and such neglect may contribute toward the persistence of amoebiasis. It appears illogical to dump large quantities of sewage into streams from which supplies of potable water must be obtained by costly methods of filtration and chemical treatment

As an illustration of several pathways that may be followed by *E histolytica* from the infected to the susceptible individual the data of Ivanhoe (33) on a children's home in New Orleans, Louisiana are of interest Using microscopic examinations, and centrifugation through a Forest centrifuge when necessary, she demonstrated cysts of *E. histolytica* on the hands, particularly under the fingernails of the children, on soiled underclothing, in the laundry chute, in sand of a playpen, and in the water and sediment of a wading pool By methods of sterilization of the premises with steam, and treatment of the children with amoebacidal drugs, the incidence of infection with *E histolytica* was reduced almost to the vanishing point

A considerable number of studies have been conducted on questions concerning the possible role of food handlers, and household servants, in the spreading of amoebiasis A discussion of the papers on this subject was omitted from the present treatise because the results are not conclusive, the affirmative statements being questioned by advocates of the theory that the disease is transmitted largely, if not exclusively, in contaminated water. Among the many papers on the role of insects, household pets, and other domestic animals, in the transmission of amoebiasis, the report by Frye and Meleney (34) indicates that the housefly may be of considerable importance

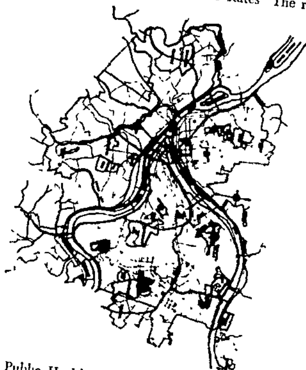
It appears that indifference to the incidence of amoebiasis and of amoebic dysentery characterizes many places in the United States Wright (35) reported that up to 1941 amoebic



Figure 1. The conquest of typhoid fever by the City of Pittsburgh, Pennsylvania. Selected to illustrate effects of methods of sanitation on a fecal-borne disease. Incidence in 1908 (*above*), and in 1930 (*opposite page*). Effective filtration and chemical treatment of water supplies were instigated in 1908 (Obtained through the courtesy of Dr C F Drake, of the Water Filtration Plant, and Drs G. R. Lacy, and T. McC. Mahon of the University of Pittsburgh, School of Medicine)

dysentery (designated under various categories) was reportable in forty-four states and the District of Columbia. In some states, cases of amoebic dysentery only were reported while in other states reports included cases of amoebiasis. In one state, reports were based on clinical evidence alone, while in 16 states diagnosis

was confirmed by laboratory examination before the case was reported Replies to a questionnaire indicated that in 13 states cases of infection disclosed by routine laboratory examination of stools were included in reports without clinical examination or reference of the report to the physician Information on these various points was not available in some states The reports of



the U S Public Health Service, 1933-1947 show 3,305 deaths from amoebic dysentery, obviously only a small proportion of those that occurred In the Northwestern states, for example, only two cases of the infection and two deaths, were reported in one year The diagnosis was probably made on the autopsy table The above account shows that there are many ways by which *E histolytica* surmounts sanitary barriers, even though it may be transmitted only when a susceptible individual ingests

fecal material from one who is infected. Although little information is available concerning the amount of such material from which the infection with *E. histolytica* may be acquired, the experiments of Rendtorff (36) indicate that infection of a human volunteer occurred following ingestion of a single cyst of *E. coli*

Discussion

From the foregoing account it is apparent that the basic problems in amoebiasis lie primarily in the fields of physiological chemistry, bacteriology and clinical medicine. As indicated in subsequent chapters the physiological approach must include methods of cytochemistry and probably of tissue culture, as well as experimental chemotherapy. There is also continuing need for training and experience in methods of microscopy. Since cultural methods are required for all of these studies there will probably be considerable reliance on methods of empiricism. There are a number of practical problems in the conquest of amoebiasis for which a background of basic research is already available There is need, however, for emphasis on the importance of amoebiasis as a problem in public health, because of the indifference among physicians and other workers in this field that appears to prevail at the present time.

Although as was emphasized many times by Dr. Robert Hegner, a problem need not be damaged by the fact that it has practical importance, the investigator whose horizon does not transcend the practical applications will sooner or later reach a condition of stalemate. The worker who attacks a problem from the standpoint of its fundamental importance will find that his answers to particular questions may lead to other questions in fields that at first hand appeared to be unrelated to the problem at hand His success will depend not only on enthusiasm and diligence but also on selection of methods of approach by which complicating factors may be eliminated, or at least reduced to a minimum.

Summary

The history of observations and researches on amoebiasis shows great diversity in the opinions concerning the role played by *Entamoeba histolytica* in the etiology of amoebiasis The early conflicting opinions were attributable largely to the paucity of experimental data, and to lack of information concerning the genera and species of amoebae that parasitize the intestinal tract of man Many of these conflicts were eliminated by the experiments of Walker, and Walker and Sellards and by cytological studies, particularly those of Dobell, showing the occurrence of four genera and five species. *Entamoeba histolytica, E coli, Endolimax nana, Iodamoeba butschlii,* and *Dientamoeba fragilis,* only the first species being demonstrably pathogenic Later controversies are concerned with questions whether *E histolytica* may live in "carriers" as a commensal, whether it is always pathogenic, or whether symbiosis of amoebae and bacteria may be required for pathogenicity. Success in 1925 in the cultivation of *E histolytica* under practicable conditions had a marked influence on these controversies Methods of approach, furnishing definitive answers to the questions are urgently needed.

Although *E histolytica* may be transmitted only when a susceptible individual ingests cysts from one who is infected, there are many questions that have not been answered concerning pathways available to the cysts in such transfers However, the installation of plants for filtration and chemical treatment of water, and elimination of many unsanitary practices, have been followed by marked reduction in the incidence of the infection

Therefore, in addition to basic problems on the metabolism of the amoeba there are many immediately practicable problems open to present-day investigators

Bibliography

1 LOESCH, F Massenhafte Entwickelung von Amoben im Dickdarm *Virchow's Arch Path Anat*, 65 196-211, 1875

2 WALKER, E L A comparative study of the amoebae in the Manila water supply, in the intestinal tract of healthy persons, and in amoebic dis- *Philippine J Sc*, 6 259 279, 1911

3 Walker, E. L., and Sellards, A. W. Experimental entamoebic dysentery. *Philippine J. Sc.*, 8 253-331, 1913.
4 Dobell, C. *The Amoebae Living in Man*. London, John Bale, Sons & Danielsson LTD, 1919, 155 pp.
*5 Hakansson, E. G. *Dientamoeba fragilis*, a cause of illness. *Am. J. Trop. Med.*, 16 175-185, 1936.
*6 Hakansson, E. G. *Dientamoeba fragilis*. Some further observations. *Am. J. Trop. Med.*, 17 349-362, 1937.
7 Cutler, D. W. A method for the cultivation of *Entamoeba histolytica*. *J. Path. & Bact.*, 22 22-27, 1918.
8 Hegner, R. W. and Taliaferro, W. H. *Human Protozoology*. New York, Macmillan Company, 1924, 597 pp.
9 Boeck, W. C. and Drbohlav, J. The cultivation of *Endamoeba histolytica*. *Am. J. Hyg.*, 5 371-407, 1925.
10 Calkins, G. N. *Biology of the Protozoa*. Philadelphia, Lea & Febiger, 1933, 607 pp.
*11 Craig, C. F. *Amebiasis and Amebic Dysentery*. Springfield, Illinois, Charles C Thomas, Publisher, 1934, 315 pp.
12 Elmassian, M. Sur une nouvelle espèce amibienne chez l'homme, *Entamoeba minuta* n. sp. *Centralbl. Bakt.*, *I. Abt.*, *Orig.*, 52 335-351, 1909.
13 Reichenow, E. In Doflein-Reichenow. *Lehrbuch der Protozoenkunde*. Jena, Germany. Verlag von Gustav Fisher. 1929, 126 pp.
14 Reichenow, E. Die Pathogenetische Bedeutung der Darmprotozoen des Menschen. *Centralbl. Bakt.*, *I. Abt.*, *Orig.*, 122 195-213, 1931.
15 Reichenow, E. Die Biologie der *Entamoeba histolytica* als Grundlage fur die Pathogenese. *Arch. Schiffs. Trop. Hyg.*, 41 257-262, 1937.
*16 Craig, C. F. Some unsolved problems in the parasitology of amoebiasis. *J. Parasitol.*, 22 1-13, 1936.
17 Faust, E. C. Amebiasis in the New Orleans population as revealed by autopsy examination of accident cases. *Am. J. Trop. Med.*, 21:35-48, 1941.
18 Anderson, H. H., Bostick, W. L., and Johnstone, H. G. *Amebiasis. Pathology, Diagnosis and Chemotherapy*. Springfield, Illinois, Charles C Thomas, Publisher, 1953. 431 pp.
*19 Hoare, C. A. Parasitological Reviews. The commensal phase of *Entamoeba histolytica*. *Exper. Parasitol.*, 1 411-427, 1952.
*20 Craig, C. F. Observations upon the hemolytic, cytolytic and complement-binding properties of extracts of *Endamoeba histolytica*. *Am. J. Trop. Med.*, 7 225-240. 1927.
21 Brumpt, E. *The Epidemiology of the Dysentery Amoeba and Related Forms*. The PHI TAU PHI Lecture Series, Canton, Lingnan Univ., Chapter, No. III 1-40, 1935-36.
22 Deschiens, R. Role de la flore microbienne associée à l'amibe dysentérique dans l'étiologie de l'amibiase expérimentale. *Compt. rend. Soc. biol.*, 125 1017-1020. 1937.
23 Deschiens, R. La biologie de l'amibe dysentérique dans ses relations avec l'amibiase. *Gaz. Med. France*, 57 Numero spec. 629-631, 1950.

24 Meleney, H E and Frye, W W Studies on *Endamoeba histolytica* and other intestinal protozoa in Tennessee V A comparison of five strains of *E histolytica* with reference to their pathogenicity for kittens *Am J Hyg*, *17* 637-655, 1933

25 Alexander, F D and Meleney, H E A study of diets in two rural communities in Tennessee in which amebiasis was prevalent *Am J Hyg*, *22* 704-730, 1935

26 Councilman, W T and Lafleur, H A Amoebic dysentery *Johns Hopkins Hosp Repts*, *2* 395-548, 1891

27 Westphal, A Betrachtungen und experimentelle Untersuchungen zur Virulenz der *Entamoeba histolytica* beim Menschen *Arch Schiffs Trop Hyg* *41* 262-280, 1937

28 Cleveland, L R and Sanders, E P The virulence of a pure line and several strains of *Entamoeba histolytica* for the liver of cats and the relation of bacteria, cultivation, and liver passage to virulence *Am J Hyg*, *12* 569-605, 1930

29 Amberg S A contribution to the study of amoebic dysentery in children *Bull Johns Hopkins Hosp*, *12* 355-363, 1901

30 Blanc F and Siclier, F L'amibiase Étude clinique et therapeutique L'Expansion Scientifique Française Preface de Lemeierre, 1950, 634 pp

31 Chiray, M and Chene, P Les formes frustes de l'amibiase hépatique *Gaz Med France*, *57* Numero spec 771-774, 1950

32 Lacy, G R History of bacteriology in hospitals Symposium 54th Ann Meeting *Soc Am Bact*, Pittsburg Pennsylvania 1954

33 Ivanhoe, G L Studies on the transmission of amebiasis in a children's home in New Orleans *Am J Trop Med*, *23* 401-419, 1943

34 Frye W W and Meleney, H E Investigations of *Endamoeba histolytica* and other intestinal protozoa in Tennessee IV A study of flies, rats, mice, and some domestic animals as possible carriers of the intestinal protozoa of man in a rural community *Am J Hyg*, *16* 729-749, 1932

35 Wright, W H The public health status of amebiasis in the United States, as revealed by available statistics *Am J Trop Med*, *30* 123-133, 1950

36 Rendtorff R C The experimental transmission of human intestinal protozoan parasites I *Endamoeba coli* cysts given in capsules *Am J Hyg*, *59* 196-208

37 Armstrong T G, Wilmot, A J and Elsdon-Dew, R The treatment of amoebic dysentery in the Bantu African *Tr Roy Soc Trop Med & Hyg*, *42* 597-601 1949

38 *Ibid* Aureomycin and amoebic dysentery *Lancet*, *259* 10-12, July 1, 1950

39 *Ibid* Terramycin in the treatment of amoebic dysentery *South African M J*, *26* 766-768 1952

40 Pichet, A Le traitement des dysenteries amibienne et bacillaire par nouveaux antibiotiques *Presse Med*, *61* 831, 1953

CHAPTER 2

DIAGNOSIS OF INFECTION WITH *ENTAMOEBA HISTOLYTICA*

Introduction

METHODS of diagnosing infection with *Entamoeba histolytica* are of paramount importance in problems of amoebiasis. At the present time the only method receiving wide attention is the microscopic examination of fecal specimens for the detection of trophic amoebae or cysts. This method is very time-consuming and requires highly trained technicians who under the most favorable conditions could not be made available in numbers sufficient to meet requirements of practicing physicians throughout the country. Examinations made by inadequately trained individuals may do more harm than good since many bodies occurring in the stool, such as macrophages, and lipoidal materials, may be mistaken for amoebae. Even by competent protozoologists stool examinations are sometimes considered significant only when positive. If a method might be developed wherein growth of the amoebae *in vitro* occurred with regularity from an inoculum of fecal material, many questions concerning the possible participation of *E. histolytica* in cases of enteritis where no specific causative agent is detectable by microscopic examination of stools, could be answered. The difficulties in perfecting such a method lie in the numerous species of bacteria occurring in the inoculum, without which the amoeba has not been induced to grow and with which it may be killed by bacterial overgrowth. The situation is not as discouraging as before antibiotics that inhibit bacteria without appreciably affecting the amoeba were made available in quantity. A promising field of investigation on methods of diagnosis of amoebiasis by culture methods has thus been provided. From the standpoint of ascertaining whether the patient may have symptoms attrib-

utable to attacks of *E histolytica* the development of immunodiagnostic methods is highly desirable A discussion of such methods is therefore indicated.

In connection with, or supplementary to, the three methods of diagnosis mentioned above, the development of a fourth method based on inoculation of laboratory animals with fecal material from the patient is needed Prior to 1948 when cats, dogs, and monkeys were the only experimental animals considered as satisfactory experimental hosts of the amoeba the use of methods of animal inoculation as a means of diagnosis was impracticable because of the difficulties of obtaining and maintaining these hosts in the laboratory However, the demonstrations by Tobie (1), Carrera and Faust (2), and Taylor *et al* (3), of fulminating infections in the rabbit or the guinea pig following intracaecal inoculations of trophozoites of *E histolytica* by the technique proposed for kittens by Meleney and Frye (4) have brightened the outlook for diagnosis by animal inoculation The use of this method is, therefore, proposed The technique of inoculation is not difficult to acquire by workers trained in methods of animal experimentation Even though animal inoculations would be quite expensive, since from five to 10 guinea pigs would probably be required for each case, and a waiting period of 20 days or longer may be required for the incubation period, the method would provide an unequivocal positive diagnosis when amoebic dysentery occurred in the experimental host Particularly in cases where all attempts by the physician to ascertain the causative agent of the patient's symptoms have failed, the method of animal inoculation would have great value For such cases a waiting period of 20 days or longer would be of little consequence

Precautions Against Infection of Laboratory Workers with Pathogens

There is lack of awareness by some diagnosticians that dangerous pathogenic bacteria and/or viruses may occur in fecal specimens and that certain of these may be transmitted to the diagnostician, to other workers in the laboratory, to visitors, and

even to plumbers The dangers from aerosols have been emphasized by Stein and associates (5) and by many other bacteriologists According to Kessel *et al.* (6) the virus of poliomyelitis occurred in the feces of a number of cases of poliomyelitis at autopsy

Although cleanliness in handling infectious material appears to be almost an inborn trait of some workers and acquired with

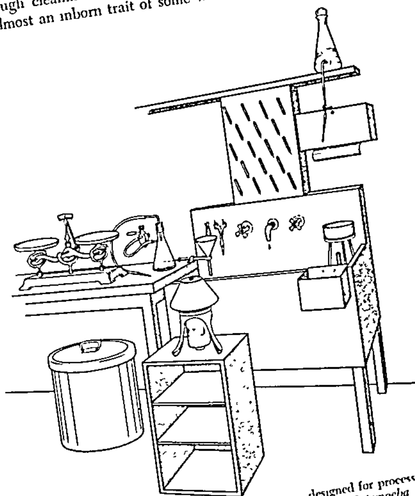


Figure 2 Working space in the writer's laboratory, designed for processin[g] fecal specimens prior to examination of concentrates for *Entamoeba h[is]tolytica*, and/or other protozoan organisms For explanation, see text.

difficulty by others, certain over-all precautions may be taken generally. The work of fecal diagnosis should be conducted in a restricted area of one room. Except under conditions of careful handling, stool specimens should not be carried to other rooms. The space allotted and equipment used in the writer's laboratory is illustrated in Figure 2. One of the most important items is the pan of water supported over a Bunsen burner in a corner of the waste sink. Immersion in hot water will kill all dangerous pathogens, with the exception of certain spore-forming bacteria. The advantages of supporting the centrifuge on a low table arise from the fact that cups and other containers need not be lifted at levels higher than the shoulders. The forceps and hemostat hung on the wall of the sink may be used for handling hot containers and also for mixing and comminuting fecal material. The usefulness of the funnel as a soap dish, and of the holder of paper towels, in positions of ready access needs no special comment. The box hooked on the side of the sink is a useful container for cotton swabs and plugs that make readily disposable cleaning pads. If at all practicable the diagnostic laboratory should be in the basement of the building, unless provision is made for direct connection of the waste sink to the sewer system. Although the bulk of fecal material should be disposed of in a garbage pail or other receptacle that may be emptied at the incinerator, some of this material is unavoidably emptied into the sink. If the sink used is in the upper stories of the building, and if special provisions were neglected in planning the plumbing system, all sinks at lower levels may be contaminated.

Diagnosis by Stool Examination

Cardboard containers with tight fitting covers, on which the name of the patient may be written at the time of passing the specimen, appear to be well suited for specimens that may be collected at or near the diagnostic laboratory. For patients from whom the specimen must be obtained at points distant from the laboratory preserving solutions have been developed for which glass jars are used. In the method of Brooke and Goldman (7)

the material is preserved in PVA fixative. It is prepared by dissolving 5 gm. (Elvanol, E. I. du Pont de Nemours & Co.) in a solution composed of 1.5 cc. of glycerol, 5 cc. glacial acetic acid, and 93.5 cc. of Schaudinn's solution (two parts saturated aqueous mercuric chloride to one part 95 per cent ethyl alcohol). The Elvanol is dissolved by heating the solution to 75° C., with stirring, until a water-clear solution is obtained. Fecal material transferred to this fixative at field stations is smeared onto a glass slide at the diagnostic laboratory, and the smear dried in air, preferably at 37° C., prior to staining. The preparation is then stained in iron hematoxylin. Trophozoites stain well, cysts appear distorted but their nuclei are frequently differentiated with sufficient clearness to permit diagnosis.

The M.I.F fixative of Sapero and Lawless (8) contains Lugol's iodine solution, 10 parts, C. P. formaldehyde 15 parts, and tincture of merthiolate (Eli Lilly and Co.) 75 parts. From fecal material thus preserved the cystic stages of amoebae may be identified without further staining if examined within several days of fixation. Addition of more Lugol's solution may improve the staining of material preserved for longer periods.

Whenever practicable the freshly passed stool should be examined in water or saline solution, and also after treatment with *D'Antoni's iodine solution* (9). (*One gram of potassium iodide* is dissolved in 100 cc. of distilled water and to this solution is added 1.5 gm. of crystalline powered iodine.) Directions for making the fecal smear were given by Craig (10) as follows:

"A small portion of the material to be examined should be picked up on a wooden toothpick, mixed with a drop of normal* salt solution, and rapidly streaked across a *microscopic slide, for* a space covering the width of two cover-slips and one-half of this smear covered immediately with a cover-slip. A drop of iodine solution, preferably D'Antoni's, is then mixed with material on the other half of the smear and this, in turn, covered with a cover-slip." The painstaking examination of such smears *should* never be neglected since a positive identification of *E. histolytica*

*8.5 gm NaCl in a liter of distilled water

finishes the diagnosis. The most needed qualification of the diagnostician is patience Not infrequently the parasite has been found through examination of the entire preparation from cases in which the technician was about to declare a negative diagnosis from examination of smaller areas

Some criteria for identification of E histolytica

A recent report by Brooke *et al* (11) of a committee of the American Society of Tropical Medicine, that was organized to obtain information from prominent protozoologists throughout the United States and Canada, concerning methods of stool examination for detection of intestinal amoebae, shows considerable disagreement concerning methods of procedure The paper summarizes replies to 115 questions on diagnosis, some 40 of which were on methods of examining fecal specimens The difficulties of differentiating among species of amoebae, on the basis of examination of trophozoites were generally recognized, and it was conceded that the cystic stage of the amoeba furnishes better criteria than the trophic stage for differentiating between *E histolytica* and other species As indicated in the preceding chapter these criteria were clearly portrayed by Dobell (12)

As pointed out in Chapter 1, *E histolytica* in its cystic and trophic stages was described and illustrated, and differentiated from other species of intestinal amoebae before methods of cultivating these parasites *in vitro* were discovered However, in the present treatise a description of the life cycle of *E histolytica* is presented in Chapter 3 since phases of its life cycle were ascertained largely from cultural studies The differentiating criteria of its cysts are based largely on the number, size, and other characteristics of the nuclei which range from one to four depending on the stage of the ripening process at which the cyst may be found In preparations stained with iron hematoxylin as well as those of unstained cysts in water the chromatoidal body has diagnostic significance but this body can usually not be seen in cysts that have been stained in iodine The cysts of *E coli* are usually larger than those of *E histolytica*, and have one to eight, and sometimes sixteen, nuclei Chromatoidal bodies are less

frequently found than in *E. histolytica* but such bodies in *E. coli* differ in size and shape from those of *E. histolytica*. In the former species they resemble chips of wood whereas in the latter they are frequently bean-shaped. *Endolimax nana*, and *Iodamoeba butschlii* may be differentiated with ease from either of the two species of *Entamoeba*. Within the cyst of *I. buetschlii* there is a large body that stains deeply with iodine, and not infrequently a similar body may be found in *E. nana* In each of the two last species the nucleus is far richer in chromatin than in species of *Entamoeba* As indicated in Chapter I, *Dientamoeba fragilis* has no known cystic stages, and except by the well-trained protozoologist, its identification may be difficult. Although it is usually described as having two nuclei, the recently divided amoeba has one nucleus. Since excellent descriptions of the five species of intestinal amoebae are available in books, particularly the monograph of Anderson *et al.* (13) further details here do not appear necessary.

The principal difficulties of the diagnostician lie in ascertaining whether frank cases of infection with *E. coli* may also have *E. histolytica* since there may be many unripe cysts of *E. coli* in the stool In this situation dependence must be placed on differences between the nuclei, the general size of the cysts, the nature and size of residual masses of glycogen, and the shape of the chromatoidals, when present. These differences are difficult to describe but may be ascertained by study of figures and through long experience in examination of unstained and stained preparations of fecal material. Special problems are encountered in borderline cases where an occasional cyst of *E. coli* having four nuclei may fit the classical description of *E. histolytica* As emphasized by Anderson *et al.* (13), the organism should be studied in fresh material and in material fixed and stained in iron hematoxylin prior to degenerative changes.

Other difficulties may arise in examinations of material from certain chronic cases of intestinal amoebiasis, and those of extraintestinal amoebiasis where the output of cysts in the stool may be scanty In a study conducted by von Brand (14) on examination of stools of a carrier case over a period of sixty days

there were four days on which no cysts could be found On other days the output of cysts ranged from 6,200 to 1,333,300 per gram of feces Based on many such experiences there is general agreement among competent diagnosticians that a negative report should not be issued until a number of stools passed on different days have been examined Some clinicians, recognizing these difficulties, administer a saline cathartic as a means of flushing out the amoebae from the bowel The principal objection lies in the occurrence of trophozoites only, in the cathartic stool and few protozoologists will issue a diagnosis of infection with *E histolytica* without evidence based on examination of cysts This reservation does not apply to frank cases of amoebic dysentery where blood and mucus and trophozoites with ingested red blood cells occur in the stools Methods of concentrating the cysts from specimens in which none may be found by direct examination are described later in the chapter

The zinc sulfate centrifugal flotation method of concentrating cysts

In 1926 Yorke and Adams (15) described a method of floating cysts of intestinal protozoa on a solution of cane sugar (sp gr 1 080) The float was separated from the heavier substances, diluted with four volumes of water, and the cysts recovered by centrifugation The sugar solution has not been used extensively by others but the method furnished a stimulus for development of the zinc sulfate flotation technique published by Faust and associates (16) about twelve years later They found that a solution of zinc sulfate (sp gr 1 180) would float cysts of *E histolytica* without demonstrably damaging them The cysts were removed in wire loops, and also by contact with glass slides or cover-slips placed on the surface of the solution Their data show that up to 69 per cent of positive cases could be diagnosed by a single flotation compared with less than half of this percentage by a direct examination of the fecal smear Certain improvements in the method with detailed directions for conducting it were published by Tobie *et al* (17) as follows

"1 With a split tongue depressor select a formed portion of the fecal specimen about the size of a large pea and place di-

rectly into a Wassermann tube (13 × 100 mm.). Standard tongue depressors are split lengthwise into three equal parts and the ends cut off square.

"2. Add 3-4 ml of tap water and thoroughly comminute the fecal material with the tongue depressor. Remove any large, undigested particles with the depressor.

"3. Shake the tube vigorously by grasping it so that the thumb covers the opening.

"4. Fill the tube with tap water to within one cm. of the top, and with the thumb over the opening of the tube invert it quickly so that an even suspension is obtained. The amount of tap water entering the tube can be regulated by merely placing the thumb partly over the opening of the tube and allowing running water to fall on the top of the thumb. By slightly rotating the thumb the opening into the tube can be increased or decreased.

"5. Centrifuge at 2,500 RPM for one minute

"6 Pour off the supernatant by inverting the tube rapidly into a waste sink. If the correct amount of fecal material has been utilized initially, there should be just enough packed sediment to cover the concavity of the tube after centrifugation.

"7. Add approximately 1.5 ml. of tap water and by holding the tube near its top with one hand, tap the bottom of the tube against the open palm of the other hand until the sediment is thoroughly mixed. Fill the tube with tap water to within one cm of the top and invert the tube as before. Great care must be exercised to make sure that the protozoan cysts or helminth eggs and larvae from one fecal tube are not transferred to another tube by the use of the thumb in the shaking procedure. This may be obviated by following the simple rule of always keeping the thumb under a running stream of water during the periods when it is not involved in the shaking procedure

"8 Centrifuge at 2,500 RPM for one minute and rapidly pour off the supernatant. The washing of feces by centrifugation should be repeated until the supernatant is relatively clear. In practice this usually requires two to three centrifugations.

"9. By means of a siphon bottle add approximately 1.5 ml. of

zinc sulfate solution (sp gr 1 180) and briskly tap the bottom of the tube against the open palm of the hand as before After the sediment is thoroughly mixed add zinc sulfate solution to within 0 5 cm of the top of the tube Do not place the thumb on the opening of the tube and invert because the parasites begin to float very soon after the introduction to the zinc sulfate and many of the organisms will be removed by the thumb if it is used as a cap for the tube. Care should be taken that the spout from the zinc sulfate bottle does not become contaminated by touching it to the top of the tube

"10 Centrifuge at 2,500 RPM for one minute All of the centrifugations should be done with a centrifuge having a head which rotates the tubes in a horizontal plane

"11 By means of a wire loop 5 mm in diameter, carefully remove three or four loopfuls of material from the surface film and place directly on a 3 1-1/2 inch glass slide If large fecal particles are floating on the surface they should be carefully pushed to one side of the tube with the wire loop before the diagnostic material is removed The wire loop should be gently inserted just below the surface film at one side of the tube, moved horizontally a short distance and drawn upward through an undisturbed portion of the meniscus Number 30-gauge picture wire is cut into a three inch length and a wire loop formed by twisting the wire around a glass rod (5 mm in diameter) The loop is then bent at an angle of approximately 75° so that the wire loop can be introduced into the Wassermann tube with ease The loops are used one time and then discarded The diagnostic material should be removed from the surface film of the various specimens as soon as possible after the organisms have been floated Otherwise, the organisms may absorb the zinc sulfate solution, sink to the bottom of the tube and not be recovered from the surface films

"12 Immediately add a drop of D'Antoni's iodine stain (9) and gently rotate the slide on the bench top to concentrate the organisms toward the center of the drop Add a 22 x 22 mm cover-slip and examine."

Zinc sulfate solution (sp gr 1.160) contains 400 gm $ZnSO_4$ per

rectly into a Wassermann tube (13 × 100 mm.). Standard tongue depressors are split lengthwise into three equal parts and the ends cut off square.

"2 Add 3-4 ml of tap water and thoroughly comminute the fecal material with the tongue depressor. Remove any large, undigested particles with the depressor.

"3 Shake the tube vigorously by grasping it so that the thumb covers the opening.

"4. Fill the tube with tap water to within one cm. of the top, and with the thumb over the opening of the tube invert it quickly so that an even suspension is obtained. The amount of tap water entering the tube can be regulated by merely placing the thumb partly over the opening of the tube and allowing running water to fall on the top of the thumb By slightly rotating the thumb the opening into the tube can be increased or decreased.

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"12 Immediately add a drop of D'Antoni's iodine stain (9) and gently rotate the slide on the bench top to concentrate the organisms toward the center of the drop Add a 22 x 22 mm cover-slip and examine"

Zinc sulfate solution (sp gr 1 180) contains 400 gm $ZnSO_4$ per

liter of distilled water, but solutions should be checked with a hydrometer. The results of a statistical analysis of data obtained by the technique described above are illustrated in Figure 3, which shows that although five flotations were required to detect all of 28 known cases of infection with *E. histolytica*, 71 per cent were detected in the first test.

The technique described above is probably the most efficient thus far devised for the fecal diagnosis of *E. histolytica*. Elsdon-

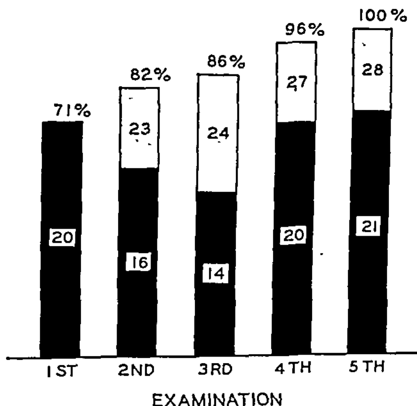


Figure 3. Results of retest by zinc sulfate centrifugal flotation of stools of 28 patients known to harbor *Endamoeba histolytica* Lower bar represents number of positive stools detected on each examination Full bar represents cumulative number of cases detected through specified examination Percentages represent cumulative per cent of cases detected through specified examination. (From. *Am J Trop Med*, *31*.558, 1951. Courtesy of the Editor.)

Dew (18) has recently reported on the effectiveness of the technique in tests on a total of 1,539 fecal specimens. His data show an over-all efficiency of 86.4 per cent for the flotation technique compared with 49.2 per cent for the direct examination of the fecal smear. The technique has been criticized, especially by bacteriologists, because of the practice of using the thumb as a cap for the Wassermann tube. A risk of becoming infected with pathogenic bacteria or viruses through the use of this technique must, therefore, be considered. However, some other method might be found for capping the tubes without appreciably slowing down the operations.

The writer has used a method based largely on a flotation technique developed by Snyder and Meleney (19) in which a tube shaped somewhat like the well-known Babcock tube for cream analysis is used for flotation. Dr Buonomini of the University of Pisa has followed our lead with the use of such a tube (Buonomini and Braccini (20)).

Figure 4 illustrating this tube was taken from their paper. Despite the objections raised by Tobie *et al.* (17) to the angle head centrifuge it has been found very effective by the writer for the preliminary washing of the fecal specimen, and has the advantage over horizontal type machines in that metal or plastic centrifuge tubes may be used without rubber pads, and that the entire interior of the angle head centrifuge may be "sterilized" with boiling water after each run. Spattering of fecal material on the housing of horizontal type machines, and contamination of rubber pads may create hazards of accidental infection, especially when the same centrifuge is used by workers in addition to those engaged in fecal diagnosis. Preliminary comminuting and washing of the fecal specimen may be carried out along lines described by Tobie *et al.* (17) except that larger samples may be used.

Figure 5 illustrates a metal tube and a strainer which the writer has used for removing undigested material and other lumps from the sample. Such strainers machined to fit snugly into the centrifuge tube may be sterilized after each use by immersion in the boiling water. In agreement with the state-

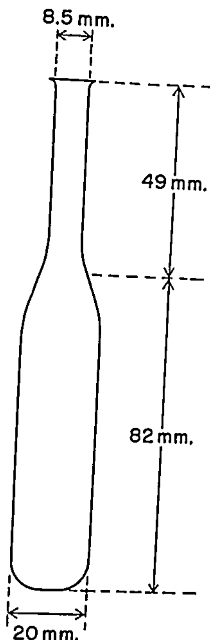


Figure 4 "Babcock" type centrifuge tube used for concentration of cysts of intestinal amoebae by zinc sulfate centrifugal flotation. (From G Buonomini and L. Braccini. *Rivista Ital d'Igiene*, 12 7, 1952 Courtesy of Authors and Editors.)

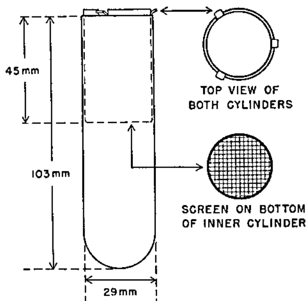


Figure 5 Sieve-bottomed "inner tube" for straining out lumps of undigested food and/or other materials from comminuted fecal specimens The centrifuge tube and "inner tube" are of metal

ment of Tobie *et al* (17) the angle head centrifuge has not been found practicable by the writer for flotation of cysts from the washed fecal suspension However, the "Babcock" type tube may be spun in the horizontal centrifuge. At the end of the spinning period of the zinc sulfate solution, the cysts may be removed from the neck of the tube by adding several cc. of water or physiologic saline, and immediately withdrawing the added liquid into a syringe The cyst-suspension in the syringe may then be transferred to a watch glass of from 50 to 150 cm in diameter, and sufficient saline solution added to almost fill the watch glass Locke's or Ringer's solution is unsatisfactory for this purpose because reactions of phosphates with the residual zinc sulfate cause clumping of the cysts in a flaky precipitate A suspension of cysts obtained by the writer's method

is illustrated in Figure 10. Although more time consuming than the method of Tobie *et al* (17) and probably therefore less suited for large scale surveys, the writer's modification of the method of Snyder and Meleney (19) is beset with fewer dangers of self infection, and of infection of other laboratory workers. As brought out in following chapters the method is also well suited for certain cultural studies on intestinal parasites.

The formalin-ether sedimentation technique

As described by Ritchie (21) the formalin-ether sedimentation technique differs from the zinc sulfate centrifugal flotation technique in that the parasites are recovered from the sediment. The preliminary operations of comminuting and washing the fecal sample are essentially the same in the two methods The washed sediment is taken up in a solution containing 10 parts of ten per cent formalin to three parts of ethyl ether and the material spun at low speed in an angle head centrifuge. The parasites are recovered from the bottom of the centrifuge tubes According to Ritchie *et al* (22) higher percentages of recoveries of cysts of *E histolytica* were obtained than by the flotation technique. Obviously the formalin-ether sedimentation technique is not usable when cultural studies on the parasites are contemplated

The value of the hematoxylin-stained smear

In an excellent chapter on "Laboratory diagnosis of amebiasis" Anderson *et al* (13) have indicated a preference for the hematoxylin-stained smear over other methods of diagnosis, especially for cases where there may be questions concerning the differentiation between *E. histolytica* and *E. coli.* An outstanding advantage of the preparation stained with iron hematoxylin over other preparations is that a permanent mount is thus obtained for verification of disputed cases. However, this statement is valid only if the material has been properly "fixed" before staining and if the preparation contains cysts that have not been allowed to deteriorate during storage of the specimen before the smear was made. If at all possible, a freshly passed stool should be called for under these conditions. Their recommendation that the technician should have or acquire skill in the delicate tech-

nique of hematoxylin staining is certainly sound. The methods of procedure selected for fecal diagnosis will depend to a considerable extent on the background and training of the investigator. Too rigid adherence to any set of directions, no matter how well stated, may retard rather than further progress toward the solution of the problems The recommendation of Anderson *et al*, that technicians should receive thorough training in a large hospital, or other laboratory, under competent supervision, before conducting fecal diagnosis, is excellent

Diagnosis by Cultural Methods

As has already been stated the principal complication in methods of cultivating intestinal amoeba from inoculum of the fecal specimen lies in the rich growth of organisms from the intestinal flora of the host The use of certain antibiotics in the cultures has lessened this complication With few exceptions the experiences of investigators of cultural methods, before the usefulness of antibiotics was demonstrated, did not favor such methods Recently, however, Eyles *et al* (23) were able to demonstrate *E histolytica* from the intestinal content of scavenger dogs by a cultural method when direct microscopic examination of the material gave negative results A good beginning toward the development of the cultural method with the use of penicillin and streptomycin has recently been made by Faust *et al* (24). To obviate difficulties of preparing the well-known whole egg medium of Boeck and Drbohlav (25) for such studies, Balamuth and Sandza (26) have developed a medium by extracting egg yolk in boiling water and enriching the cleared solution with liver extract and rice powder This medium is now available commercially Nelson (27) has also prepared a wholly liquid medium by extracting egg yolk and/or other materials in alcohol Other complications of cultural methods lie in the fact that amoebae growing out in the medium may be commensals rather than *E histolytica* Further progress in this field may, therefore, depend on better methods than now available for inducing encystation of amoebae *in vitro* The attractiveness of the problem on cultural methods is enhanced rather than diminished by the prospects of studying encystation

Immunodiagnosis

Immunodiagnostic methods for clinical amoebiasis have been conducted with antigens prepared from amoebic lesions, from amoebae grown *in vitro*, and also from cysts. Wagener (28) demonstrated antibodies in the serum of experimentally infected cats by a precipitin test with antigen prepared from scrapings of intestinal lesions, and Craig (29, 30) demonstrated antibodies in the serum of man by the complement fixation test with antigen prepared from alcoholic extraction of amoebae from cultures with a complex flora. A good review of the investigations with antigens prepared from such cultures by extraction of the amoebae in alcohol, or water, was published by Magath and Meleney (31). They also obtained data on the complement fixation test with antigens prepared by several methods of extraction. Serum specimens from a total of ninety patients were tested, all of whom either had amoebiasis at the time of the test or were known to have harbored *E. histolytica* at some previous times. Magath's laboratory and Meleney's laboratory conducted tests on serum samples from all ninety patients without consultation until the tests were completed. A total of thirty-two positive tests was obtained with agreement between the laboratories on seventeen cases. The cases giving positive tests were those showing the most severe symptoms. In view of discrepancies between the results of the two laboratories, and between their tests and those of others to whom some of the samples were sent, and particularly because of inability to standardize the antigen, they concluded that considerable improvement in the preparation of antigen will be necessary before the test may become popular.

In an early paper on the complement fixation test with antigen prepared from *E. histolytica*-organism *t**, Rees *et al.* (32) stated that "The performance of the complement fixation test for

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* "organism *t*" because when grown aerobically on blood agar medium [illegible] colonies are *transparent*

amoebiasis has not been sufficiently reliable to establish its general use in the diagnosis of this infection The limiting factor of greatest concern has been the inadequacy of the antigen, due in part at least, to numerous undetermined species of bacteria and their metabolic products which have occurred in the cultures of the specific organisms used for antigen production. To obviate as many of the complications as possible we have used cultures with a single species of bacterium"

In addition to our original study of the complement fixation test, work with antigens from *E histolytica*-organism *t* was reported by Bozicevich *et al* (33), Kent and Rein (34), Terry and Bozicevich (35), Bozicevich (36), Dolkart and Hedges (37), Hussey and Brown (38), Dolkart *et al* (39), and Kenney (40) The most encouraging report was by Kenney who obtained positive tests on 80 per cent of patients passing trophic amoebae, 28 per cent on those passing cysts, and only 3 5 per cent on those having other intestinal protozoa without *E histolytica* The least encouraging results were reported by Dolkart *et al* (39) Over a period of six years complement fixation tests were conducted on a total of 458 of Dr Dolkart's patients, on whom stool examinations conducted at his laboratory showed that sixty had *E histolytica* at the time of drawing the serum Only nineteen of the sixty had positive tests and 106 with negative stools had positive tests However, the complement fixation tests were conducted by two different laboratories of the U S Public Health Service during periods of experimentation with antigens. One laboratory reported much higher percentages of positive tests than the other It is not improbable that some of the 106 patients may have had amoebiasis at some previous times, since all had intestinal complaints

A preliminary report on the complement fixation test with antigen prepared from *E. histolytica-Bacterium coli** was published by Fulton *et al* (41) showing that a high percentage of persons harboring *E. histolytica* showed positive tests The reports of Terry and Bozicevich, and Hussey and Brown show that

*Classified in *Bergey's Manual* as *Escherichia coli*

high percentages of those having amoebic hepatitis and/ amoebic liver abscess gave positive tests. However, the stat ment of Magath and Meleney (31) that considerable improv ment of the antigen is necessary applies to all tests thus far co ducted. As stated in C[illegible]
of obscure etiology is [illegible]
to symptomatology an [illegible]
The possibilities of using other methods than the complemen fixation test require investigation. In this connection some in teresting experiments by Cole and Kent (42) indicate th *E. histolytica* may be immobilized in hyperimmune serum pro duced in the rabbit by infection of cultures of *E. histolytica* *Trypanosoma cruzi*.

Discussion

The foregoing account on methods of diagnosis by microscopi examination of fecal specimens, methods of cultivating th amoeba therefrom, and immunodiagnostic methods shows tha all have shortcomings It is not improbable that *E. histolytica* may exhibit low-grade parasitism at certain times and thus escap detection despite the most painstaking efforts of the diagnosti cian A new method based on inoculation of rabbits or guinea pigs with fecal material from suspected cases is proposed, with- out as yet any experimental data on its effectiveness, although from the time of Loesch to the present the experimental animal has been used to demonstrate pathogenicity of the amoeba.

Summary

Methods of diagnosis of infections with *E. histolytica* are diffi- *cult, time consuming, and require* the services of well-trained protozoologists with much experience in *this field.*

Careless handling of fecal material that may result in spreading infection with dangerous parasites to laboratory workers should be avoided

The unstained and iodine-stained smear of the freshly passed stool should be examined first because cases of amoebic dysentery may usually be thus diagnosed quickly. A *demonstration of cysts*

of *E. histolytica* in carrier cases constitutes a positive report. Failure to find cysts of *E. histolytica* in the fecal smear does not warrant a negative report, since methods of concentration by flotation and/or sedimentation, are practicable.

The use of the PVA and/or MIF fixative is recommended for stool specimens that must be collected at points distant from the diagnostic laboratory.

As a substitute or supplement of the methods of microscopic examination of stools development of practicable methods of diagnosing infections by cultural methods holds considerable promise since antibiotics that inhibit bacterial growth without affecting *E. histolytica* have become available. In this connection there is need for more information concerning factors of encystation of *E. histolytica in vitro*.

The complement fixation test for clinical amoebiasis using antigen processed from *E. histolytica* may furnish valuable information, particularly in cases of amoebic hepatitis or amoebic liver abscess.

Bibliography

1. Tobie, J. E. Experimental infection of the rabbit with *Endamoeba histolytica*. Am. J. Trop. Med., 29:859-870, 1949.
2. Carrera, G. M. and Faust, E. C. Susceptibility of the guinea pig to *Endamoeba histolytica* of human origin. Am. J. Trop. Med., 29:647-667, 1949.
3. Taylor, D. J., Greenberg, J., Highman, B. and Coatney, G. R. Experimental infection of guinea pigs with *Endamoeba histolytica*. Am. J. Trop. Med. 30:817-828, 1950.
4. Meleney, H. E., and Frye, W. W. Infection of kittens with *Endamoeba histolytica* by direct injection of cultures into the ileum. *Proc. Soc. Exper. Biol. & Med.* 30:277-279, 1932.
5. Stein, L., Anderson, R. E. and Gross, N. H. Potential infectious hazards of common bacteriological techniques. Soc. Am. Bact. Abst. of papers, 49th Meet. p. 10, 1949.
6. Kessel, J. F., Moore, F. J., Stimpert, F. D. and Fisk, R. T. Occurrence of poliomyelitis virus in autopsies, patients and contacts. *J. Exper. Med.*, 74:601-609, 1941.
7. Brooke, M. M. and Goldman, M. Polyvinyl alcohol fixative as a preservative and adhesive for protozoa in dysenteric stools and other liquid materials. J. Lab. & Clin. Med. 34:1554-1560, 1949.
8. Sapero, J. J. and Lawless, D. K. The "MIF" stain-preservation technique for the identification of intestinal protozoa. *Am. J. Trop. Med. & Hyg.*, 2:613-619, 1953.

9 D'ANTONI, J S.. Standardization of the iodine stain for wet preparations of intestinal protozoa Am J Trop Med, 17.79-84, 1937.

10 CRAIG, C F Laboratory Diagnosis of Protozoan Diseases Philadelphia, Lea & Febiger, 1942, 349 pp

11 BROOKE, M M, OTTO, G, BRADY, F, FAUST, E C, MACKIE, T. T and MOST, H An analysis of a memorandum on the diagnosis of amoebiasis Am J Trop Med & Hyg, 2 593-612, 1953

12 DOBELL, C The Amoebae Living in Man London, John Bale, Sons & Danielsson, Ltd, 1919, 155 pp

13 ANDERSON, H H, BOSTICK, W. L. and JOHNSTONE, H G. Amebiasis Pathology, Diagnosis and Chemotherapy Springfield, Illinois, Charles C Thomas, Publisher, 1953, 431 pp

14 VON BRAND, T Quantitative Untersuchungen uber die Zystenausscheidung von Entamoeba histolytica und Lamblia intestinalis bei einem gesunden Dauerausscheider Centralbl. Bakt, I. Abt, Orig, 123 358-365, 1932

15 YORKE, W and ADAMS, A R. D. Observations on Entamoeba histolytica I Development of cysts, excystation, and development of excysted amoebae in vitro Ann Trop Med, 20 279-302, 1926.

16 FAUST, E C, D'ANTONI, J S, ODOM, V, MILLER, M. J, PERES, C, SAWITZ, W, THOMEN, L F., TOBIE, J and WALKER, J H A critical study of clinical laboratory technics for the diagnosis of protozoan cysts and helminth eggs in feces Am J Trop Med, 18 169-183, 1938.

17 TOBIE, J E, REARDON, L V, BOZICEVICH, J, SHIH, B C, MANTEL, N and THOMAS, E H The efficiency of the zinc sulfate technic in the detection of intestinal protozoa by successive stool examinations Am J Trop Med, 31 552-560

18 ELSDON-DEW, R Zinc sulfate flotation of faeces Tr Roy Soc Trop Med & Hyg, 41 213-218, 1947.

19 SNYDER, T L and MELENEY, H E.. The excystation of Endamoeba histolytica in bacteriologically sterile media Am J Trop Med, 21 63-73, 1941.

20 BUONOMINI, G and BRACCINI, L.. Su la diagnosi parassitologica di amebiasi Nota I Due utili technihe per la ricerca delle cisti nelle feci Riv ital d'Igiene, 12 3-12, 1952

21 RITCHIE, L S An ether sedimentation technique for routine stool examinations Bull U S Army Med Dept, 8 326, 1948

22 RITCHIE, L S, PAN, C and HUNTER, G. W., III A comparison of the zinc sulfate and MGL (Formalin-Ether) technics J. Parasitol, 38 4, Sec 2, p 16, 1952.

23 EYLES, D E, JONES, F. E, JUMPER, J R and DRINNON, V. P · Amebic infections in dogs J Parasitol, 40 163-166, 1954

24 FAUST, E C, LEWIS, R, HEILBRUNN, I, HICKMAN, M L M, and EVERRITT, M G Posibles determinantes de la patogenicidad de la Endamoeba histolytica. I Estudio de sus asociaciones bacterianas y su susceptibilidad a las antibióticos Rev Méd, Mexico, 28 206-210, 1948

25. BOECK, W C and DRBOHLAV, J: The cultivation of Endamoeba histolytica Am J Hyg, 5 371-407, 1925.

26 BALAMUTH, W and SANDZA, J G Simple, standardized culture medium for physiological studies on *Entamoeba histolytica* *Proc Soc Exper. Biol & Med*, 57 161-163, 1944

27. NELSON, E C Alcoholic extract medium for diagnosis and cultivation of *Endamoeba histolytica* *Am J Trop Med*, 27 545-552, 1947

28 WAGENER, E H A precipitin test in experimental amoebic dysentery in cats *Univ Calif Publ in Zool*, 26 15-20, 1924

29 CRAIG, C F Observations upon the hemolytic, cytolytic and complement-binding properties of extracts of *Endamoeba histolytica* *Am J Trop Med*, 7 225-240, 1927

30 CRAIG, C F Complement fixation in the diagnosis of infections with *Endamoeba histolytica* *Am J Trop Med*, 8 29-37, 1928

31 MAGATH, T B and MELENEY, H E The complement fixation reaction for amoebiasis Comparative tests performed by two laboratories *Am J Trop Med*, 20 211-238, 1940

32 REES, C W, BOZICEVICH, J, REARDON, L V and JONES, F A preliminary note on the complement fixation test for amoebiasis with antigens prepared from *Endamoeba histolytica* grown with a single species of bacteria *Am J Trop Med*, 22 581-586, 1942

33 BOZICEVICH, J, HOYEM, H M and WALSTON, V M A method of conducting the 50 per cent hemolysis end point complement-fixation test for parasitic diseases *Public Health Rep*, 61 529-534, 1946

34 KENT, J F and REIN, C R The serodiagnosis of amoebiasis *Science* 103 598, 1946

35 TERRY, L L and BOZICEVICH, J The importance of the complement fixation test in amebic hepatitis and liver abscess *South M J*, 41 691-702, 1948

36 BOZICEVICH, J Immunological diagnosis of parasitic diseases, in *Parasitic Infections in Man* H Most, Ed, New York, Columbia University Press 1951, 229 pp

37 DOLKART, R E and HEDGES, R N The present incidence of intestinal infestation with *E histolytica* and other protozoa in the Chicago area *Gastroenterology*, 9 170-176, 1947

38 HUSSEY, K L and BROWN, H W The complement fixation test for hepatic amebiasis *Am J Trop Med*, 30 147-154, 1950

39 DOLKART, R E, HALPERN, R and CULLEN, J The diagnosis of amebiasis the role of the complement fixation test, and the incidence of the disease in the Chicago area *J Lab & Clin Med*, 38 804, 1951

40 KENNEY, M Micro-Kolmer complement fixation test for amebiasis *Am J Trop Med & Hyg* 1 717-726 1952

41 FULTON J D, JOYNER, L P and ORPWOOD PRICE I N Studies on Protozoa Part IV A complement-fixation test for amoebiasis *J Trop Med & Hyg* 54 27-33, 1951

42 COLE, B A and KENT, J F Immobilization of *Endamoeba histolytica in vitro* by antiserum produced in the rabbit *Proc Soc Exper Biol & Med*, 83 811-814, 1953

9 D'Antoni, J S. Standardization of the iodine stain for wet preparations of intestinal protozoa. *Am. J Trop Med*, 17.79-81, 1937.

10 Craig, C F *Laboratory Diagnosis of Protozoan Diseases* Philadelphia, Lea & Febiger, 1942, 349 pp

11 Brooke, M M, Otto, G, Brady, F., Faust, E. C, Mackie, T. T. and Most, H An analysis of a *memorandum on the diagnosis of amoebiasis* *Am J Trop Med. & Hyg*, 2 593-612, 1953.

12 Dobell, C *The Amoebae Living in Man.* London, John Bale, Sons & Danielsson, Ltd, 1919, 155 pp.

13 Anderson, H H, Bostick, W. L. and Johnstone, H G. *Amebiasis: Pathology, Diagnosis and Chemotherapy* Springfield, Illinois, Charles C Thomas, Publisher, 1953, 431 pp

14 von Brand, T Quantitative Untersuchungen uber die Zystenausscheidung von *Entamoeba histolytica* und *Lamblia intestinalis* bei einem gesunden Dauerausscheider. *Centralbl Bakt, I Abt, Orig*, 123 358-365, 1932.

15 Yorke, W and Adams, A R D Observations on *Entamoeba histolytica* I Development of cysts, excystation, and development of excysted amoebae *in vitro* *Ann Trop. Med*, 20 279-302, 1926

16 Faust, E. C, D'Antoni, J S., Odom, V, Miller, M J., Peres, C., Sawitz, W, Thomen, L F, Tobie, J and Walker, J H: A critical study of clinical laboratory technics for the diagnosis of protozoan cysts and helminth eggs in feces *Am. J Trop Med*, 18 169-183, 1938

17 Tobie, J E, Reardon, L V, Bozicevich, J, Shih, B C, Mantel, N and Thomas, E H The efficiency of the zinc sulfate technic in the detection *of intestinal protozoa by successive stool examinations. Am. J. Trop Med*, 31 552-560

18 Elsdon-Dew, R Zinc sulfate flotation of faeces *Tr. Roy Soc. Trop Med & Hyg*, 11 213-216, 1947.

19 Snyder, T L and Meleney, H E. The excystation of *Endamoeba histolytica* in bacteriologically sterile media *Am J. Trop Med*, 21 63-73, 1941

20 Buonomini G and Bracceni, L Su la diagnosi parassitologica di amebiasi Nota I *Due utili technicche per la ricerca delle cisti nelle feci. Riv Ital d Igiene*, 12 3-12, 1952

21 Ritchie, L S An ether sedimentation technique for routine stool examinations *Bull U S Army Med Dept*, 8 326, 1948

22 Ritchie, L S, Pan, C. and Hunter, G W, III. A comparison of the zinc sulfate and MGL (Formalin-Ether) technics *J Parasitol*, 38 4, Sec. 2, p 16, 1952

23 Eyles, D E, Jones, F. E, Jumper, J R and Drinnon, V. P: Amebic infections in dogs *J. Parasitol*, 40 163-166, 1954

24 Faust, E C, Lewis, R, Heidbreder, I, Hickman, M L. M and Everritt, M G Posibles determinantes de la patogenicidad de la *Endamoeba histolytica*. I Estudio de sus asociaciones bacterianas y su susceptibilidad a los antibióticos *Rev Méd, México*, 28 200-210, 1948

25 Boeck, W. C and Drbohlav, J: The cultivation of *Endamoeba histolytica* *Am J Hyg*, 5 371-407, 1925

26 BALAMUTH, W and SANDZA, J. G Simple, standardized culture medium for physiological studies on *Entamoeba histolytica* Proc Soc Exper Biol & Med, 57 161-163, 1944

27 N- son E C Alcoholic extract medium for diagnosis and cultivation of ... Trop Med, 27 513-532, 1947

28 ... entery in cats ...

29 CRAIG, C F Observations upon ... plement-binding properties of extracts of *Endamoeba histolytica* Am J Trop Med, 7 225-240, 1927

30 CRAIG, C F Complement fixation in the diagnosis of infections with *Endamoeba histolytica* Am J Trop Med, 8 29-37, 1928

31 MAGATH, T B and MELENEY, H E The complement fixation reaction for amoebiasis Comparative tests performed by two laboratories Am J Trop Med, 20 211-238, 1940

32 REES, C W, BOZICEVICH, J, REARDON, L V and JONES, F A preliminary ... on the complement fixation test for amoebiasis with antigens prepared ... with a single species of bacteria Am J

... LSTON, V M A method of conduct ... ant complement-fixation test for para... sitic diseases Public ... I 529-534, 1948

34 KENT, J F and REIN, C R The serodiagnosis of amoebiasis Scienc... 103 598, 1946

35 TERRY, L L and BOZICEVICH, J The importance of the complement fixati... test in amebic hepatitis and liver abscess South M J, 41 691-702 19...

36 BOZICEVICH, J Immunological diagnosis of parasitic diseases, in *Parasitic I... fections in Man* H Most, Ed, New York, Columbia University Press 19... 229 pp

37 DOLKART, R E and HEDGES, R N The present incidence of intestinal in... fection with *E histolytica* and other protozoa in the Chicago area Gas... enterology, 9 170-176, 1947

38 HUSSEY, K L and BROWN, H W The complement fixation test for he... amebiasis Am J Trop Med, 30 147-154, 1950

39 DOLKART, R E HALPERN, B and CULLEN, J The diagnosis of ameb... the role of the complement fixation test, and the incidence of the de... in the Chicago area J Lab & Clin Med, 38 804, 1951

40 KENNEY, M Micro-Kolmer complement fixation test for amebiasis A... Trop Med & Hyg 1 717-726 1952

41 FULTON J D JOYNER, L P and ORRWOOD PRICE I N Studies on Pro... Part IV A complement fixation test for amoebiasis J Trop Med & ... 54 27-33 1951

42 COLE, B A and KENT, J F Immobilization of *Endamoeba histolytica* i... by antiserum produced in the rabbit Proc Soc Exper Biol & ... 83 811-814, 1953

CHAPTER 3

THE USEFULNESS OF CRUDE CULTURES OF *ENTAMOEBA HISTOLYTICA*

Introduction

In the present treatise, cultures of *Entamoeba histolytica* such as those developed by Boeck and Drbohlav are considered as "crude" because all measurable metabolic activities are attributable to the many species of bacteria derived with the amoeba *from the original inoculum, and in many cases augmented by* bacterial contamination during serial transfer. On the other hand, certain cultures developed during the past 20 odd years, of the amoeba with a single selected species of bacteria, a trypanosome, or minced embryonic tissues, are designated as "monoxenic," a term proposed by Dougherty (1). The impact of the accomplishments of Cutler (2), and Boeck and Drbohlav (3) on *the thinking of investigators of amoebiasis was considered in* Chapter 1, and the usefulness of crude cultures in diagnosis by microscopic and immunodiagnostic methods was discussed in Chapter 2. Significant accomplishments with use of crude cultures in depicting all phases of the life cycle of the amoeba, in ascertaining the effects of chemical agents on cysts of *E. histolytica* in potable water supplies, in sewage, on leafy vegetables, and in other situations where *human feces may be deposited* are depicted in the present chapter. In addition some consideration *is given to the limitations* of work with such cultures in studies on infectivity and pathogenicity of the parasite. Further reference to such limitations in the above fields as well as in experimental chemotherapy is reserved for subsequent chapters

The medium of Boeck and Drbohlav (3) is herein designated as "diphasic," because it consists *of a solid phase overlaid with a* liquid phase. However, in crude cultures certain ingredients of *both phases* are *rapidly* degraded by activities of the flora so

that the nutrients utilized by the amoebae are probably products of bacterial metabolism as well as bodies of the bacteria In the preparation of media precise measurements of ingredients, even of the mineral salts, does not appear to be important. Ringer's solution, and Ringer-Locke's solution (without dextrose), as well as "normal" sodium chloride (0 85 per cent sodium chloride in water), have been used without demonstrable differences in growth of the amoebae The original formula of the liquid phase included serum but as shown by Drbohlav (4) and by later workers, liquid egg white or egg albumin may be substituted for serum Frye and Meleney (5) used liver extract instead of serum, and Reardon and Rees (6) demonstrated that enrichment of this phase is unnecessary According to Dobell and Laidlaw (7) the only improvement of the "Boeck" tube consisted in enrichment with rice starch Cleveland and Collier (8) and Cleveland and Sanders (9) introduced rice flour, prepared from whole unpolished rice, instead of starch, and such flour has been used by many subsequent workers, especially in the United States These investigators also used liver-infusion agar instead of the egg-slant However, in our experience the agar-slant gives less satisfactory results than the egg-slant because the line of demarcation between the solid and liquid phases is somewhat indefinite and not infrequently the agar slant floats to the surface of the liquid phase However, the agar slant may be prepared with less difficulty than the egg or serum slant, the medium is available commercially, and is used routinely by a number of workers in other laboratories Dobell and Laidlaw (7) used bases of inspissated serum as well as of egg and recommended that inspissation of the serum be conducted at temperatures below 100 C but egg slants may be prepared in the autoclave with the ports closed to prevent escape of air during coagulation The Media Preparation Section of the National Institutes of Health subjects the egg emulsion to a partial vacuum prior to inspissation to prevent formation of bubbles in the slant

For maintenance of the amoebae a temperature at or slightly below 37 C is required Some wholly liquid media have served

well for routine cultural studies, and for diagnosis from material Such media have been used by Snyder and Meleney (10), Balamuth and Sandza (11), and Nelson (12). They are prepared by extracting whole egg, egg yolk, and/or certain other protein substances in water or in alcohol. Except in cases of overgrowth of the amoebae by a fungus *Blastocystis*, or by what Dobell called "starch splitters," the cultures may be maintained indefinitely by serial transfer at from forty-eight to seventy-two hours of incubation. In crude cultures the amoebae has always failed to grow in nutrient broths or digests used routinely for the cultivation of bacteria.

Significant Accomplishments with Crude Cultures

Life cycle of E histolytica

From the painstaking observations of Dobell (13), and of Cleveland and Sanders (9), descriptions and illustrations of the phases of the life cycle of *E. histolytica* have been furnished. In addition to growth of the trophic amoebae these phases include encystation, excystation, and metacystic development. During encystation the nucleus within a rounded up amoeba divides twice with production of four very small nuclei compared with the mother nucleus. In many cases a body called the chromatoidal, because it takes nuclear stains, develops within the cyst and disappears at later stages that have not been clearly ascertained. Although of great importance in the microscopic recognition of the species the chemical nature and even the function of the chromatoidal body are unknown. A major research problem thus awaits attention of biochemists and/or physiologists. Excystation consists in the hatching of a fournucleate, metacystic, amoeba from the cyst. By a series of nuclear and cytoplasmic divisions eight tiny amobulae are produced from each four-nucleate amoebae during metacystic development. These developments are reminiscent of gametogenesis in certain other protozoan organisms but sexual phenomena in *E. histolytica* have never been demonstrated. An interpretation of the significance of metacystic development in *E. histolytica* is

hallenging problem In many crude cultures encystation, ystation, and metacystic development are of frequent oc- rence No reports of encystation in monozenic cultures have ne to the writer's attention On the other hand, as explained subsequent chapters, excystation has been produced in bac- a-free medium under partial anaerobiosis in the presence of rient materials, especially glucose

n contrast with the clear cut descriptions of developments ing encystation, excystation, and metacystic developments, details concerning the transitional stages from the tiny oebulae to the comparatively large trophic forms occurring the lumen of the bowel or in the tissues have been furnished cording to early viewpoints discussed in Chapter 1, the oebula attacks and destroys an epithelial cell and grows and ltiplies with production of larger trophozoites However, ac- ding to a theory that is rapidly gaining ground at the present ne, the transitional stages between amoebulae and larger forms cur within the lumen. The demonstrated occurrence in crude tures of all phases of the life cycle furnishes strong presump- e evidence for the latter theory With rare exceptions, in- stigators have attempted to prove theories or hypotheses and ve appeared unwilling to "follow the argument" regardless of outcome. However, the complications inherent in the ex- rimental approach to the problem, by inoculating amoebae m crude cultures into the bacteria-infested intestinal tract of laboratory animal, are formidable

fects of chemical agents, heat, and drying on cysts

Since chlorine and/or ozone are widely used in the treatment potable water supplies, experiments on the effects of these ents on cysts of *E histolytica* are indicated The standard pro- dure prior to the demonstrated usefulness of the cultural ethod was to treat the cysts with dilute staining solutions, ch as eosin, on the theory that only dead cysts would thus stained From experiments on the cultivation of cysts follow- g treatment with chlorine, Stone (14) concluded that when due count was taken of residual organic matter, which may neu-

tralize the chlorine, the cysts of *E. histolytica* are not more resistant than *Escherichia coli.* He recommended further work under conditions in the field. According to Chang and Fair (15) chlorine dosage and residuals are functions of temperature of the water, contact time, and pH. They concluded that the concentration of chlorine needed to destroy cysts lies within the limits of superchlorination. However, since cysts of *E. histolytica,* as well as those of *E. coli* and of other species of intestinal amoebae, may be effectively removed from municipal water supplies by methods of filtration, the practical importance of experiments on the cysticidal effects of chlorine and/or ozone lies in the treatment of relatively small supplies of water, especially for members of the armed services in war time, and also for individual use by residents or travelers in poorly sanitated places. Brady *et al.* (16) were concerned with these situations. They used water in Lyster bags of a capacity of thirty-five gallons, added hypochlorite tablets, and calculated numbers of cysts of *E histolytica.* They recovered the cysts after measured periods of exposure, and ascertained whether amoebae could be obtained therefrom in whole egg medium that was seeded also with a standard bacterial flora. The water was taken from a stream furnishing a municipal water supply prior to its filtration and chlorination Treatment for thirty minutes with two hypochlorite tablets per bag prevented growth of most of the cysts. They concluded that considerable, though not complete, protection was thus afforded to those who might drink the water.

The data of Kessel *et al* (17) were obatined on the comparative cysticidal effects of chlorine and ozone. They stated that: "The bactericidal and cysticidal times required by ozone producing a residual of 0.3 p.p.m. were several times less than those required by chlorine at from 0.5 to 1.0 p.p.m." They inoculated from 800 to 1,000 cysts per tube of medium but Newton and Jones (18) showed that effects of ozone may be more accurately determined from an average inoculum of 10 cysts per tube. However, in agreement with Kessel *et al* they found that ozone at 0.3 p.p.m. during exposure for five minutes killed the cysts.

Several products available commercially in tablet form, including Halazone* and Aqua-tabs* which contain chlorine and Globaline*, containing iodine, have been recommended for use in small quantities of water by travelers in poorly sanitated places However, the palatability of the water is seriously affected by these products, particularly those containing chlorine. Their cysticidal effects under short-term exposure are limited Until better cysticidal agents become available for use under the conditions indicated, the traveler might well drink only safe bottled water, water that has been heated above 50 C, or hot beverages

The lethal temperature for cysts was ascertained by Chang (19), and by Jones and Newton (20) Application by Chang of the Arrhenius equation, and other mathematical formulae, indicated that the activating energy required to kill cysts is at or near 50 C, the temperature calculated also for inactivation of protein The data of Jones and Newton show that exposure for five minutes at 50°C, provides an adequate margin of safety. The work of Andrews (21) using the eosin staining technique indicated that cysts of *E histolytica* deposited in feces under fingernails could withstand the effects of drying However, Reardon *et al* (22) using bacteria-free cysts, demonstrated that *E histolytica* was killed by drying

Beaver and Deschamps (23) did pioneer work on the comparative effects of acetic, hydrochloric, and other acids, on cysts that had been deposited on leafy vegetables Their work shows that acetic acid is far more cysticidal in comparable strengths than hydrochloric acid In further work on these problems, Jones (24) paid strict attention to the temperature as well as to times of exposure and to the effects of treatment on palatability of vegetables immediately after treatment, as well as after periods of storage of the treated products in the ice box Her work shows that the use of strong vinegar, and/or salad dressing containing five percent acetic acid affords considerable protection against

*Halazone tablets are listed in Merck's Index Aqua-tabs and Globaline are sold by Wallace & Tiernan Products Inc., Belleville, New Jersey

infection with *E. histolytica*. However, strengths of the acid at temperatures sufficient to kill all of the cysts had deleterious effects on the vegetables.

Limitations of the Usefulness of Crude Cultures

The association or symbiosis of *E. histolytica* with bacteria of crude cultures furnishing the inoculum has *in many cases been* the only practicable method of producing experimental infection of laboratory animals with the amoeba. However, little information has been obtained on the basic pathogenicity of the amoebae because its activities have been masked by those of bacteria. These questions, as well as those on metabolism of the amoeba, are considered in some detail in later chapters but it is pertinent to point out here that methods of approach with cultures of the amoeba furnishing material that may be suitable for analysis are urgently needed in studies on infectivity, pathogenicity, experimental prophylaxis and chemotherapy.

Discussion

The theory that cysts that are alive do not stain in dilute solutions of eosin is open to question. It has been tacitly assumed, however, that cysts failing to excyst in culture are dead. In recent years, on the other hand, it has become increasingly apparent that the criteria of life or death of a microorganism are difficult to ascertain For example, the work of Hemmets *et al.*, (25) indicates that a number of metabolic functions may be carried on by bacteria that fail to grow out in culture following treatment with formalin and other bactericidal substances. The question whether a cyst of *E. histolytica that fails to excyst in vitro* may nevertheless excyst *in vivo* requires consideration. It has been *assumed, without adequate data, that the cysts pass* unharmed through the stomach of the host, resist the actions *of digestive juices in the upper* small intestine, and excyst in the vicinity of the ileocaecal valve The factors of excystation within the intestinal tract of the host are, however, unknown. Later work may show that conclusions concerning the killing power of chemical agents, heat, and drying, on cysts of this amoeba may

need revision. In any event, there is need of further work on the problems in question by methods of approach having fewer complicating factors.

Summary

Cultures of *E. histolytica* grown in the presence of a complex flora have been designated as crude in contradistinction to monoxenic cultures of the amoeba with selected single species of a bacterium, a trypanosome, or of embryonic metazoan cells.

In earlier work on cultivation, excepting that of Cutler, the media used were diphasic, with a solid phase of egg, serum, or other material coagulated by heat, supplemented with starch or rice flour and a liquid phase of a saline solution enriched with serum, egg albumin, or liver extract, or without enrichment. In recent years some liquid media enriched with starch or flour have been used.

The life cycle of *E. histolytica* has been described and illustrated through observations and experiments. Although the phases including encystation, excystation, and metacystic development, have been clearly depicted, the transitional stages between the metacystic amoebulae to the trophic forms in the intestinal tract of the host and in cultures has not been followed in detail.

Significant achievements with the use of cultures have been obtained on the cysticidal effects of chlorine, ozone, acetic acid, and of heat and drying. These studies are of practical importance in the treatment of relatively small quantities of water, and of leafy vegetables that may be contaminated with human feces.

Formidable complications have attended experiments on infectivity, pathogenicity, and chemotherapy of *E. histolytica* in laboratory animals inoculated with amoebae in crude cultures.

Bibliography

1. DOUGHERTY, F. C. Problems of nomenclature for the growth of organisms of one species with and without associated organisms of other species. Parasitology, 42:259-261, 1953.
2. CUTLER, D. W. A method for the cultivation of *Entamoeba histolytica*. *J. Path. & Bacteriol.* 22:22-27, 1918.

3 BOECK, W. C and DRBOHLAV, J. The cultivation of *Endamoeba histolytica*. *Am J Hyg*, 5 371-407, 1925.

4 DRBOHLAV, J Une nouvelle preuve de la possibilité de cultiver *Entamoeba dysenteriae* type *histolytica* *Ann. Parasitol*, 3 349-357, 1925.

5 FRYE, W W. and MELENEY, H E. Liver extract as a substitute for serum in *the culture medium for Endamoeba histolytica* *Science*, 69 564-565, 1939

6 REARDON, L V and REES, C W. The cultivation of *Endamoeba histolytica* without serum *J Parasitol*, 25 13-14, 1939

7 DOBELL, C and LAIDLAW, P P.. On the cultivation of *Entamoeba histolytica* and some other entozoic amoebae. *Parasitology*, 18 283-318, 1926

8 CLEVELAND, L R and COLLIER, J: Various improvements in the cultivation of *Entamoeba histolytica* *Am J Hyg*, 12 606-613, 1930.

9 CLEVELAND, L. R and SANDERS, E P.: Encystation, multiple fission without encystment, excystation, metacystic development, and variation in a pure line and nine strains of *Entamoeba histolytica* *Arch. Protistenk*, 70 223-266, 1930.

10 SNYDER, T L and MELENEY, H E.: *Anaerobiosis and cholesterol as growth* requirements of *Endamoeba histolytica*. *J. Parasitol*, 29 278-284, 1943

11 BALAMUTH, W. and SANDZA, J. G. Simple, standardized culture medium for physiological studies on *Entamoeba histolytica* *Proc. Soc Exper. Biol & Med*, 57 161-163, 1944

12. NELSON, E C Alcoholic extract medium for the diagnosis and cultivation of *Endamoeba histolytica* *Am J Trop. Med*, 27 545-552, 1947.

13 DOBELL, C Researches on the intestinal protozoa of monkeys and man I General introduction, and II Description of the whole life-history of *Entamoeba histolytica* in cultures. *Parasitology*, 20 357-412, 1928

14 STONE, W S The resistance of Endamoeba histolytica cysts to chlorine in aqueous solutions *Am J. Trop Med*, 17 539-551, 1937.

15 CHANG S L and FAIR, G M. *Viability and destruction of the cysts of Endamoeba histolytica* *J Am Water Works A*, 33 1705-1715, 1941

16 BRADY, F J, JONES, M F *and* NEWTON, W L. *Effect* of chlorination of water on viability of cysts of *Endamoeba histolytica* *War Med*, 3 409-419, 1943

17 KESSEL, J F, ALLISON, D K, KAIME, M, QUIROS, M and GLOECKNER A. The cysticidal effects of chlorine and *ozone on cysts of Endamoeba histolytica*, together with a comparative study of several encystment media *Am J Trop Med*, 24 177-183, 1944

18 NEWTON, W F and JONES M F. The effect of ozone in water on cysts of *Endamoeba histolytica*. *Am J Trop Med*, 29 669-681, 1949

19 CHANG, S L [illegible] cysts of *Endamoeba histolytica* in wa[illegible]

20 JONES, M F [illegible] [illegible]sts of *Endamoeba histolytica* in water at temperatures between 45° and 55°C *Am J Trop Med*, 30 53-58, 1950

21 ANDREWS J The retention of *Endamoeba histolytica* cysts under fingernails *Am J Trop Med*, 14 439-441, 1934

22 REARDON, L. V, VERDER, E and REES, C. W: The cultural requirements of *Endamoeba coli* and the comparative effects of drying on the cysts of *E coli* and *E histolytica* *Am J Trop Med & Hyg*, 1 155-161, 1952

23 BEAVER, P C. and DESCHAMPS, G The effect of acetic acid on the viability of *Endamoeba histolytica* cysts *Am J Trop Med*, 29 193-197, 1949
24 JONES, M F Studies on treatment of fresh vegetables contaminated with cysts of *Endamoeba histolytica* I Acetic acid *Am J Trop Med & Hyg*, 1 570-584, 1952
25 HEINMETS, F, TAYLOR, W W and LEHMAN, J J The use of metabolites in the restoration of the viability of heat and chemically inactivated *Escherichia coli* *J Bacteriol*, 67 5-12, 1954.

CHAPTER 4

THE PROBLEM OF ELIMINATING COMPLICATIONS ATTRIBUTABLE TO GROWTH OF SPECIES OF BACTERIA IN CULTURES OF *ENTAMOEBA HISTOLYTICA*

Introduction

As pointed out in Chapters 2 and 3 the usefulness of crude cultures of *Entamoeba histolytica* is limited by complications attributable to growth of a number of species of bacteria with the amoebae. These complications were encountered and appreciated by the early users of the Boeck and Drbohlav cultures. In 1926 Dobell and Laidlaw (1) stated these complications as follows "Cultivation of the amoebae by methods involving such a number of unknown variables—which sort themselves out, in the end, by chance—is obviously unscientific. Success often depends on luck. It is, therefore, desirable to discover some means of controlling the composition of the bacterial flora at will, but this we have found to be extremely difficult." Since that time, however, a number of methods have been developed for eliminating the flora from the amoeba, or from its cysts, and replacing the flora with a selected single species of a microorganism. An account of these methods constitutes the subject matter of the present chapter.

Bacteria-free Amoebic Liver Abscess

A bacteria-free abscess in the liver resulting from infection with *E. histolytica* does not contain pus as usually defined in medical literature since there is little if any granulocytopoiesis. Bacteria-free abscesses were produced in the liver of cats by Cleveland and Sanders (2, 3) by injecting this organ with amoebae and bacteria. The stock culture used for the inoculum was obtained from a single cyst by the following method: Cyst suspensions

were washed as free as possible from bacteria by centrifugation, stored for six days at 1° to 3°C, and cysts removed from the suspension in platinum loops to pieces of cover-slips. The droplets on the cover-slips were examined microscopically, with transfer to medium of those containing only one cyst. No mention was made of the number or species of bacteria occurring in the pure line thus produced but the flora was undoubtedly simplified in comparison with that occurring in the original culture from which the cysts were obtained. From a total of 109 cats that were injected with amoebae from the pure line culture, 11 bacteria-free amoebic liver abscesses were reported. From inoculation of medium with abscess-material, and seeding it with *Bacillus brevis* a monoxenic culture of *E. histolytica* was obtained. They established monoxenic cultures with five additional species of bacteria, and noted that six other species would not support growth of the amoeba.

Freeing Cysts from Bacteria by Chemical Treatment

Cleveland and Sanders reported occasional "sterilization" of cysts of *E. histolytica* through treatment with mercuric chloride. Meleney *et al* (4) eliminated bacteria from suspensions of cysts by this method and Snyder and Meleney (5, 6) obtained a monoxenic culture. The cysts previously washed and concentrated by the zinc sulfate centrifugal flotation technique were "sterilized" by treatment for fifty minutes in a 1-50,000 solution of mercuric chloride. Dobell (7) obtained a monoxenic culture of the amoeba from cysts that were stored for up to three weeks in the refrigerator in a dilute solution of acriflavine, removed from storage to medium containing gentian violet, and seeded with *Bacterium** *coli*. The above methods of chemical treatment were employed prior to the discovery of the usefulness of antibiotics in eliminating bacteria from cultures of amoebae. The antibiotic approach is discussed later.

***Escherichia coli*, according to *Bergey's Manual*

Microisolation

To obviate the time-consuming features of methods described above for eliminating bacteria from cultures of the amoeba, the writer developed a method of microisolation (Rees, 8). A micromanipulator of simple design and construction was used, patterned after those described in the *pioneer work of Barber* (9). As illustrated in Figure 6 our manipulator may be constructed from the base of a *discarded microscope*, with a mechanical stage attachment, and other pieces of paraphernalia either already present in the laboratory or obtainable from local sources. Although our micromanipulator does not have the fine precision of those of Chambers, Taylor, or de Fonbrunne (Chambers and

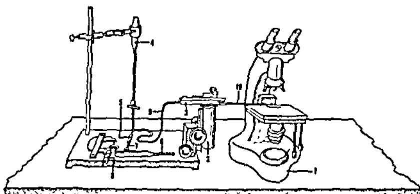


Figure 6 Micromanipulator for isolation of cysts of intestinal amoebae and other protozoan organisms from bacteria. Numbers 1, 2, and 3 are self-explanatory 4 Mercury reservoir. 5 Tuberculin syringe. 6, 7, 8 Assembly for control of plunger of syringe. 9. Capillary plastic tubing 10. Micropipette (*Reproduced through the courtesy of Dr. Angus M Griffin.* The George Washington University, School of Medicine)

Kopac, 10), it has been used effectively for isolation of cysts of *E. histolytica from bacteria as well as of trophozoites* (11, 12). The movements backwards and forwards of the meniscus in the *pipette are effected by forces of mercury induced in a capillary* tube by a tuberculin syringe. In a modification of our design by Griffin (13), these movements are controlled by a vernier caliper, a lever and a coiled spring surrounding the plunger-

shaft as illustrated in Figure 6. We have used a modification of a design by Fitz (Chambers and Kopac, 10) as shown in Figure 7

The nipple of the syringe is attached to the horizontal arm of an inverted T of glass tubing, the vertical arm of which contains a stopcock and a mercury reservoir as shown in Figure 6 The method of using the technique in routine studies is illustrated in Figures 9 and 11 The distal horizontal arm is attached to a piece of capillary plastic tubing The distal end of the plastic tubing is attached to a piece of thick-walled pyrex glass tubing

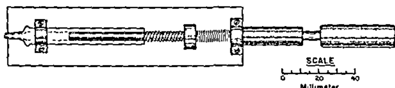


Figure 7 Assembly patterned after the Fitz micromanipulator, for controlling the plunger of a tuberculin syringe for use in manipulation (Courtesy of Bausch and Lomb Optical Company)

of approximately 2 mm bore, which serves also as the micropipette holder All connections mentioned above between glass are effected with the use of glass blowers' wax A wax sold for depilation under the trade name of ZIP is excellent for this purpose A wax seal is also used between the micropipette holder and the butt of the micropipette as illustrated in Figure 6 Control of the movements of the tip of the micropipette in three dimensions is effected by the mechanical stage and the coarse adjustment of the microscope stand The entire assembly is mounted on a base of wood, metal, or plastic material and may thus be moved manually to and from the vicinity of the research microscope Filling of all tubular spaces of the manipulator with mercury, free from bubbles, requires considerable practice and skill with precautions against breakage of the tubing

Operations requiring considerable skill are concerned with making, mounting and drawing out the tip of the micropipette Soft glass tubing of approximately 9 mm o d may be heated over a flame, which must be hotter than a Bunsen burner, pulled out

to a diameter of approximately 2 mm. o.d and a segment selected that fits snugly into the lumen of the micropipette-holder. The tip of the pipette is made by drawing out the distal end over a microburner. Such a burner may be made by leading the gas from the laboratory mains through a steel bleeding needle. The method of making the tip was described by Barber (9) as follows:

"Lower the flame of the microburner to a narrow blue flame of not over 2 mm. high. The smallest flame that will remain lighted should be used, and the working table should be free from drafts of air. Hold the shank of the pipette in the right hand, and with a pair of forceps held in the left, grasp the capillary at a point about 6 cm. from the shank. The outer sides of both hands should rest on the table. Bring the portion of the

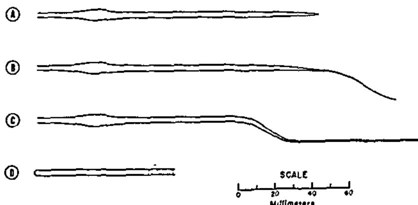


Figure 8 A Micropipette with properly drawn-out tip B. Improperly made tip. C Micropipette for insertion of organisms into microtubes D Micro-culture with petrolatum seal.

capillary next to the forceps over the flame and at right angles to it, then lower it to a point above but not in the flame. Pull gently with the forceps, and when the glass begins to soften, lift it slowly from the flame and pull with the forceps slightly more than at first, but not too strongly. The hands should remain on the table during the process and the pulling done by turning them slowly outward. The capillary will separate with a slight

tug, a feeling much like that experienced when a taut thread held in the fingers, is parted in a small flame" If the point is properly made, it will appear as in Figure 8A For microisolation of cysts the lumen of a properly made tip must be slightly enlarged This may be accomplished by touching the tip very gently with a glass slide



Figure 9 Method of removing mercury from a contaminated micropipette prior to its removal and replacement

Barber (9) and most other micrurgists have selected micro-organisms from hanging drops in which case a moist chamber must be provided and the tip of the pipette bent upward instead of downward For isolating cysts of *E. histolytica*, hanging drops are unnecessary since the cyst-suspension sinks rapidly to the bottom of a flat dish from which cysts may be selected by the micropipette under low magnification Since cysts must be passed through several changes of sterile 0 85 per cent sodium chloride to clear them of bacteria, several changes of the micropipet

of antibiotics having inhibitory or killing effects on certain species of bacteria. The use of certain antibiotics has provided a means of inhibiting bacteria in cultures of *E. histolytica* without demonstrably affecting the amoebae. Jacobs (15) eliminated *Clostridium perfringens* with the use of penicillin from a culture of the amoeba obtained by microisolation with this bacterium, and maintained the amoebae through several months by adding heat-killed *Escherichia coli*. Certain antibiotics have, however, been found to affect the amoebae The occurrence of species of parasitic fungi, which is not uncommon in crude cultures of the amoeba, renders the antibiotic approach untenable There may also be species of bacteria in such cultures that are not affected by any antibiotics thus far discovered. *Pseudomonas aeruginosa*, known also as *Bacillus pyocyaneus*, has in our hands survived in the presence of a number of antibiotics at levels that may be tolerated by protozoan parasites

Discussion

It is pertinent to compare the various techniques described above as to their usefulness in obtaining monoxenic cultures of *E histolytica* and as methods of approach in the problem of obtaining axenic cultures. The production of bacteria-free amoebic liver abscesses and the method of eliminating bacteria with the use of chemical compounds, are too time consuming, and too uncertain of outcome for practicable usage. In our hands the microisolation technique has had neither of these drawbacks, especially when used in conjunction with certain antibiotics. However, up to the present time, the method has found limited usage in other laboratories. Faust *et al.* (16) have indicated a preference for other techniques on the ground that microisolation requires special skill and is time-consuming However, their data on the use of antibiotics as a means of obtaining cultures of *E. histolytica*-organism *t* indicate that considerable time was spent with relatively unsatisfactory results From the stools of twenty-one individuals harboring *E. histolytica* they obtained cultures from only twelve. Transplants of five of the twelve strains were treated with 5,000 units of penicillin, and 10,000

of streptomycin per ml of the medium with the result that only one monoxenic culture was reported. The method selected by the investigator will obviously depend on his background and training There appears no good reason for using the technique of microisolation in cases where the bacteria of the cultures are sensitive to known antibiotics. On the other hand, where the problem involves elimination of species of fungi, and/or certain antibiotic-resistant species of bacteria, the technique of microisolation may prove to be invaluable The need for special skill does not appear an adequate criterion for rejecting this technique Special skill is required for many important operations in microbiology.

Summary

Species of bacteria may be eliminated from cysts of *E histolytica* occurring in stools of infected individuals, or in harvests of cultures *in vitro*, and sometimes from trophozoites, by one of the following four methods, or combinations of methods (1) Establishment of bacteria-free liver abscesses in experimental animals following injection of the amoeba and bacteria, and transferring abscess material into medium (2) Treatment of washed cyst-suspensions with mercuric chloride or alternately with acriflavine and gentian violet. (3) Microisolation of cysts and sometimes of trophozoites (4) Treatment of cultures of the amoeba and bacteria with antibiotics

The methods of liver-abscess production and of chemical treatment of cysts are time consuming and of uncertain outcome Microisolation is a very efficient method but requires considerable skill and training The antibiotic approach has thus far not received adequate trial It is effective insofar as the species of bacteria and/or fungi occurring with the amoebae may be sensitive to the antibiotics selected

Bibliography

1 DOBELL, C and LAIDLAW, P. P: On the cultivation of *Entamoeba histolytica* and some other entozoic amoebae. *Parasitology, 18* 283-318, 1926.

2 CLEVELAND, L. R. and SANDERS, E P.. The virulence of a pure line and several strains of *Entamoeba histolytica* for the liver of cats and the relation of bacteria, cultivation, and liver passage to virulence *Am. J Hyg, 12* 569-605, 1930

3 CLEVELAND, L. R and SANDERS, E. P.. The production of bacteria-free amoebic liver abscesses in the liver of cats and observations on the amoebae in various media with and without bacteria *Science, 72* 149-151, 1930

4. MELENEY, H E, FRYE, W. W, LEATHERS, W. S and SNYDER, T. L: The sterilization of the cysts of *Endamoeba histolytica*, with preliminary observations on subsequent excystation *Proc 3rd. Internat Cong Microbiol*, 410-411, 1940

5 SNYDER, T L and MELENEY, H. E: The excystation of *Endamoeba histolytica* in bacteriologically sterile media. *Am J Trop Med, 21* 63-73, 1941.

6 SNYDER, T L and MELENEY, H E.: Anaerobiosis and cholesterol as growth requirements of *Endamoeba histolytica J. Parasitol, 29* 278-284, 1943

7 DOBELL, C An improved method for testing the action of emetine and other chemicals on *Entamoeba histolytica* in cultures *Ann. Soc Belge Med Trop*, (Liber Jubilaris, J Rodhain) 201-211, 1947.

8 REES, C W The construction of a micromanipulator for the isolation of protozoa *Am J Trop Med, 22*,487-492, 1942

9 BARBER, M. A The pipette method in the isolation of single microorganisms and in the inoculation of substances into living cells *Philippine J. Sc*, *4* 307-385 1914

10. CH [illegible]

Ed, 1937

11. CHINN, B D, JACOBS, L, REARDON, L V. and REES, C W.: The influence of the bacterial flora on the cultivation of *Endamoeba histolytica Am J Trop. Med*, *22* 137-146, 1942

12 PHILLIPS, B P. Comparative effects of certain species of Trypanosomidae on the growth of *Endamoeba histolytica* in the absence of bacteria *Am J Trop Med*, *31* 290-294, 1951.

13 GRIFFIN, A M Microisolation technique in protozoology, with special reference to the construction of a simplified micromanipulator. Manuscript.

14. REES, C W, REARDON, L. V and BARTGIS, I L: The excystation of *Entamoeba histolytica* without bacteria in microcultures *Parasitology, 40* 338-342, 1950

15 JACOBS, L The elimination of viable bacteria from cultures of *Endamoeba histolytica* and the subsequent maintenance of such cultures *Am J Hyg*, *46* 172-176, 1947

16 FAUST, E C, LEWIS, R, HEILBRUNN, I, HICKMAN, M. L. M and EVERRITT, M G Posibles determinantes de la patogenicidad de la *Endamoeba histolytica* I Estudio de sus asociaciones bacterianas y su susceptibilidad a los antibióticos, *Med Rev. Mex*, *28* 206-210, 1948

CHAPTER 5

THE USEFULNESS OF MONOXENIC CULTURES OF *ENTAMOEBA HISTOLYTICA* IN PROBLEMS ON GROWTH REQUIREMENTS

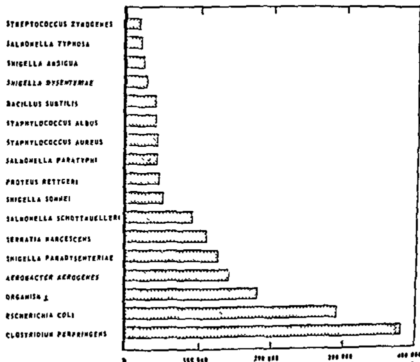
Introduction

IN previous chapters the usefulness and limitations of crude cultures of *Entamoeba histolytica* have been considered. Although many of these limitations apply also to monoxenic cultures, even the one developed by Shaffer and Sienkiewicz (1), and Shaffer *et al* (2), without any associated microorganisms, the experimental work that has been conducted has added appreciably to our knowledge of the growth requirements and metabolism of the amoeba, and represents progress toward the goal of obtaining axenic growth of the parasite. These accomplishments are considered in the present chapter.

Media and Methods of Cultivation

In our early experiments with cultures of *E. histolytica* developed by microisolation of cysts with selected single species of bacteria it was of interest to ascertain which species will and which will not support growth of the amoeba (Chinn *et al*, 3) along lines initiated earlier by Cleveland and Sanders (4). In a review of this subject by Jacobs (5) with some original data, he pointed out that species supporting, and those not supporting, such growth have been classified in widely separated taxonomic groups, the inference being that the identification of the factors is a formidable problem. By the use of hemocytometer counts on harvests of monoxenic cultures of the amoebae we have shown that quantitative as well as qualitative differences occur among the factors affecting growth (Rees *et al*, 6). Our data are illustrated in Figure 12 which shows a more than ten-fold increase

in numbers of amoebae obtainable with *Clostridium perfringens* over those with certain other species of bacteria, including species that produce bacillary dysentery The problem of estimating the growth of bacteria in these cultures is difficult. Although some information may be obtained from measurements of turbidity the work is complicated by some cloudiness attributable to the growth of the amoeba



We have obtained some information on the growth of the bacterium by measuring gas production (Rees *et al*, 7). As illustrated in Figure 13 gas-measuring cylinders were prepared by sealing pieces of glass tubing of small bore to the bottom of test tubes, and passing the tubes through rubber stoppers inserted into the culture tubes. The volume of liquid displaced into the

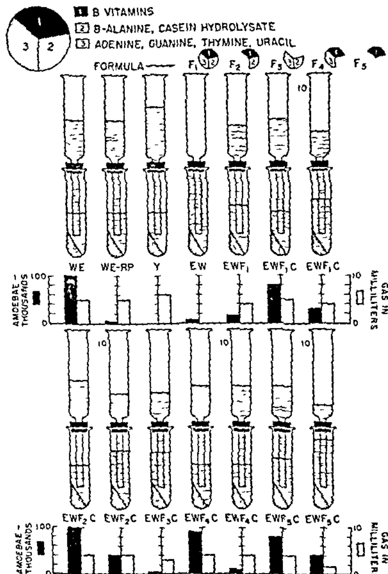


Figure 13. Numbers of amoebae and amounts of gas produced from cultures of *Entamoeba histolytica*-organism t in medium prepared from whole egg (WE), egg yolk (Y), or egg white (EW). All media were enriched with rice powder except (WE-RP). The egg white medium was used without other enrichment or with indicated formulae plus or minus cholesterol (C). The original inoculum was from whole egg medium and the results shown either at the first or the tenth serial transfer (10).

cylinder during incubation was a rough measure of the amount of gas produced. Figure 13 shows high yields of amoebae and gas in the complete whole egg medium, and high gas production with practically no amoebae when rice flour was omitted from the formula High production of gas occurred also, with no growth of amoebae, in medium prepared from egg yolk both with and without an enrichment formula. Medium prepared from egg white, with and without cholesterol, produced neither amoebae nor gas, but produced considerable gas when enriched with a formula of B vitamins and other growth factors. This formula plus cholesterol (AF_1C) produced good growth of amoebae and high levels of gas when the inoculum was derived directly from whole egg medium but not on indefinite serial transfer The figure shows also that of all the formulae used none was more effective than (AF_5C) which contained B vitamins and cholesterol. Although cholesterol was not essential for growth of organism *t* (AF_1) this compound was required by the amoebae even in the presence of rice flour manufactured from unpolished rice, and therefore containing compounds closely resembling cholesterol, but probably in very small amounts Although the data point strongly to a requirement of cholesterol the work exemplifies difficulties in ascertaining growth requirements of the amoeba

The data illustrated in Figure 14 on the comparative growth of *E histolytica* in egg white medium with organism *t* and with *Escherichia coli* show that the latter species supported growth of the amoeba through indefinite serial transfer even without enrichment with B vitamins (Rees *et al.*, 6). *E coli* is known to produce B vitamins and our work indicates that it produces unknown factors such as those present in whole egg, lacking in egg white, and not produced by organism *t*. The presumptive evidence is that *E histolytica* may require B vitamins. Some other data having a bearing on these questions also have been obtained (7, 8, 9) The data on cholesterol are in confirmation of those reported earlier by Snyder and Meleney (10).

Our experiments have shown that certain amoeba-producing ingredients of medium are present in small amounts When

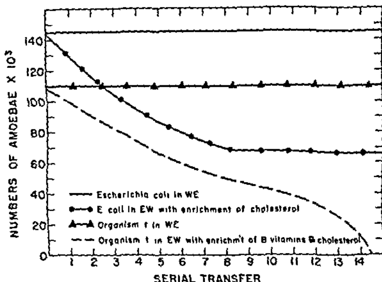


Figure 11 Growth of *Endamoeba histolytica* with *Escherichia coli*, and with organism *t* in whole egg (WE) and egg white media (EW) (From *Am J Trop Med & Hyg*, 2 1006, 1953 Courtesy of the Editor)

egg-slants that had produced one crop of amoebae were resterilized, reoverlaid, and reseeded with *E. histolytica*-organism *t* there was practically no growth of amoebae without evidence that the egg base had been appreciably degraded (Rees and Reardon, 12) The work of Jacobs (11) in which for the first time *E histolytica* was grown *in vitro* without living bacteria indicates that only traces of factors supplied by the companion microorganism may be required Heat-killed *Escherichia coli* plus ingredients supplied by the medium served to support some growth of the amoebae for several months until apparently some carry-over material or enzyme derived from an early seeding with *Clostridium perfringens* became exhausted through serial transfer The clostridia were eliminated with the use of penicillin In a long series of experiments (Rees and Reardon, 12) it was impossible to obtain consistent harvests of amoebae in wholly liquid media prepared from peptones of meat, soya bean,

peanut or cotton seed that were seeded also with organism *t*. Measurements of gas suggested, however, that failure of the amoeba to grow was attributable to overgrowth by the bacterium. At that time the usefulness of antibiotics as inhibitors of growth of bacteria was unknown. A reopening of the problem with the use of these agents is indicated. However, with transplants obtained from us, Hansen (13), Hansen and Anderson (14), and Hallman *et al.* (15) have succeeded in cultivating *E. histolytica*-organism *t* in wholly liquid medium containing amino acids, liver extract, and rice flour. Preliminary work by Hansen (13) indicated also that l-cysteine, methionine, cholesterol, and liver extract with proteose-peptone, provided a suitable medium for *E. histolytica*-organism *t*.

Additional information on this subject was furnished by Shaffer and Frye (16) and Shaffer *et al.* (17). A species of bacterium called "streptobacillus," but not as yet generically or specifically identified, was grown overnight in thioglycollate medium plus dextrose (Baltimore Biological Laboratory), then partially cleared by centrifugation, and used as a component of medium with serum. To this medium was then added a calculated amount of penicillin, and an inoculum of amoebae from a previous transfer. A petrolatum seal was added to retard entry of oxygen into the medium during incubation Excellent growth of the amoeba was obtained with little demonstrable activity by the bacterium. However, filtrates of the bacterial culture, and/or other products of bacterial metabolism, have not been successfully substituted for the living "streptobacillus." The data show that factors supplied by the living bacterium are essential for growth of the amoeba. Sadun *et al* (18), in continuing efforts to attain the goal of associate-free cultivation of the amoeba, have substituted amniotic and allantoic fluids of chick embryos for some of the ingredients of the medium of Shaffer and Frye. Phillips (19) obtained cultures of *E. histolytica-Trypanosoma cruzi* by substituting this trypanosome for a bacterium His method is similar to that of Shaffer and Frye. *T. cruzi* is grown by itself at or near 20 C. in diphasic blood agar medium, harvested at seventy-two hours or later, and added to medium with an inoculum of a

previous transplant of the amoeba culture Antibiotics may be used during initiation of the culture when derived initially from cultures with bacteria but their use thereafter is not necessary. Cultures of *E histolytica-T cruzi* are also readily obtainable from microisolated cysts (Phillips *et al*, (20)) The work of Phillips and Rees (21) shows that the living trypanosome must be present for growth of the amoeba although some growth of amoebae was obtained with suspensions of the trypanosome that had been heated up to five minutes at 48 C. From one trophic amoeba that was isolated per tube into microcultures an average progeny of ten was obtained when the trypanosome suspension had been previously heated compared with a progeny of thirty-two amoebae with trypanosomes not heated Other investigations showed (22, 23) that the amoeba did not grow with other species of *Trypanosoma* closely related to *T cruzi* or with species of *Leishmania* or *Trichomonas* Phillips' work (24) shows that although *T cruzi*, inactivated at 48 C would not grow in NIH diphasic medium, and showed no oxygen uptake in the Warburg respirometer, it reduced methylene blue, and produced acid Suspensions heated as high as 50 C produced no such activities Therefore, factors supporting growth of *E histolytica* appear to be thermolabile at about the temperature required to inactivate protein Suspensions of the trypanosome that were disintegrated by freezing-thawing, treatment with distilled water, hydrochloric acid, or ether, did not furnish requirements for growth of the amoeba In the dialysate medium of Tobie and Rees (25) the amoebae would not grow with active *T cruzi* probably because the concentration of the trypanosomes was too low About 3 $\times$ 10^7 trypanosomes per ml of the medium have been found necessary for good growth of the amoeba The work of Shaffer and Sienkiewicz (1) shows that an atmosphere free from oxygen is required for growth of *E histolytica* in their chick-embryo medium Consumption of oxygen may be an important contribution of *T cruzi* toward growth of the amoeba but if this were the only factor the amoeba should grow in cultures with other species of *Trypanosoma* and with *Leishmania* Ingestion by the amoebae of *T cruzi* and of the other species of

Trypanosomidae as well as species of *Trichomonas* has been noted in our experiments, so that the question whether phagocytosis of the associated organism furnishes food for the amoeba could not be answered However, the data on cultures in chick embryo mince point strongly toward the theory that *E. histolytica* is a phagotroph, since the trophozoites appear filled with these cells, or cellular detritus and have not grown unless proportionately large quantities of the mince were used.

We have attempted to cultivate *E. histolytica* in medium supporting bacteria-free excystation (Rees *et al.*, 26). As illustrated in Figure 15 a medium containing a balanced salt solution enriched with glucose, B vitamins, vitamin C, purines, pyrimidines, and other ingredients including cysteine or glutathione brought about high percentages of bacteria-free excystation in sealed microcultures. However, not a single case of division of the amoeba hatching from the cysts was noted unless another species of microorganism was added. A long series of investigations in collaboration with Dr. Hellerman of The Johns Hopkins University (27) in which the medium was fortified with all available coenzymes failed to reveal the factors required for associate-free growth of the amoeba. The preceding discussion shows that associate-free cultivation of *E. histolytica* may furnish an attractive problem for a team of investigators including protozoologists, bacteriologists physicists, biochemists, and physiologists.

Preliminary Experiments on Metabolism

An analysis of some experiments on the metabolism of *E. histolytica* was recently furnished by M. Lwoff (28). Of particular interest is her discussion of oxidation-reduction studies by von Brand *et al* (29, 30), Rees and Reardon (8), Chang (31), Bradin and Hansen (32), and Jacobs (33). Von Brand *et al.* (29) showed that reducing substances in medium seeded with *E. histolytica*-organism *t* dropped to low levels during the first twenty-four hours of incubation, and that production of gas, principally hydrogen, was also effective in lowering the oxygen content of the medium These results were attributed to growth of organism *t* Rees and Reardon showed that the amoeba-

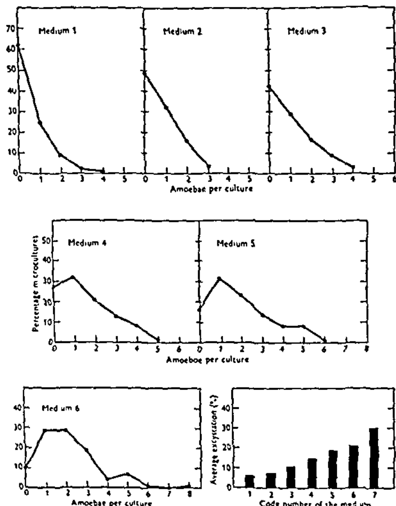


Figure 15 Effects of organic compounds added to inorganic solutions plus a reducing agent on percentages of excystation of *Entamoeba histolytica* without bacteria in microcultures Media (1) inorganic compounds without bicarbonates plus cysteine (2) inorganic compounds including bicarbonates plus cysteine, (3) same as (1) plus glucose, (4) same as (2) plus glucose (5) same as (1) plus glucose, B vitamins, Vitamin C, cocarboxylase, purines, pyrimidines, folic acid, glucosamine, cholesterol, and amino acids (6) same as (5) with glutathione substituted for cysteine (7) same as (5) without amino acids (From *Parasitology, 40* 240, 1950 Courtesy of the Editor)

producing potential of sterile egg white medium is lost during storage in air but not in containers filled completely with medium and having an airtight seal, also that amoebae would grow with organism *t* in diffusible substances from the egg white base consisting largely of ovomucoid. Potentiometric studies by Chang indicated that the amoeba multiplied at O-R levels below –150 mv., that *excystation* occurred at about –115 mv. and that a rise in potential considerably above –115 mv. was required for encystation. His data were obtained from crude cultures and are not in agreement in several respects with those of Jacobs on monoxenic cultures, where multiplication of the amoeba occurred at levels as high as –40 mv. Jacobs commented as follows. "Any medium supporting growth of *Entamoeba histolytica* is a mixture of many ingredients; hence . . . Eh values cannot be interpreted in terms of the proportion of oxidized and reduced forms of a particular substance. Moreover, the relationship between Eh and concentration of oxygen in the medium is also a matter of speculation." He concluded that "certainly anaerobiosis can no longer be considered the most neglected factor" in the problem of ascertaining the requirements for pure culture of *E. histolytica.* On the whole, the data on oxidation-reduction favor the theory that *E. histolytica* belongs with the anaerobes but formidable complications have been encountered in cultures with bacteria.

Some quantitative data have recently been obtained on carbon dioxide production from cultures of *E. histolytica* with single species of bacteria, compared with such production by the bacteria grown without amoebae (Rees *et al*, *6*). Some of these data are presented in Table I.

Two modifications of an Eldredge tube (*6, 34*) in which these cultures may be grown and carbon dioxide measured are illustrated in Figures 16 and 17. Increased production of the gas in the presence of the amoebae was determined by increased amounts of standard N/10 acid required to neutralize barium hydroxide dispensed in the "boat" of the Eldredge tube, using phenolphthalein as indicator. Except in one set of cultures wherein glucose was added the whole egg medium in which

TABLE I

COMPARATIVE DETERMINATIONS OF CARBON DIOXIDE AND TURBIDITY FROM *Endamoeba Histolytica-Aerobacter Aerogenes and A. Aerogenes* IN ELDREDGE TUBE CULTURES AT 96 HOURS OF INCUBATION

E. histolytica-A. aerogenes					*A. aerogenes*		
5-tube set of cultures	Amoebae per tube X 10^4	CO_2 in cc	Turbidity Log Io/I	CO_2/T	CO_2 in cc	Turbidity Log Io/I	CO_2/T
1	57.5	3.69	0.515	7.2	2.13	0.396	5.4
2	56.0	4.72	0.552	8.5	1.90	0.325	5.8
3	52.0	4.70	0.573	8.2	1.85	0.382	4.9
4	52.0	4.87	0.537	9.1	1.85	0.390	4.7
Average	51.4	4.49	0.544	8.3	1.94	0.374	5.2
5*	.	14.78	1.210	12.2	16.32	1.010	16.1

*Enriched with glucose in the amount of 1 per cent

The figures on carbon dioxide were corrected for changes in titer of the barium hydroxide attributable to absorption of carbon dioxide from the air as ascertained by titrations of sterile medium

From *Am J Trop Med and Hyg*, 2 1010, 1953. (Courtesy of the Editor)

cultures of amoeba-bacterium, and bacterium, were grown contained only as much sugar as diffused from the egg base, approximately 0.4 M/10^3. A theoretical explanation for increased amounts of carbon dioxide in the presence compared with that in the absence of amoebae is that the amoeba liberated substrate from rice flour which was utilized by the bacterium with resulting richer growth of the latter. Fragmentation of the flour with liberation of starch grains is illustrated in Figure 18 which shows also that the bacterium by itself did not fragment the rice. Our data (Rees *et al*, 6) show that the amoebae alone or in symbiosis with the bacterium, liberated protease which liquefies gelatin and probably fragments the rice. Recently Hallman and DeLamater (35) have demonstrated amylase from *E. histolytica* and their data have been confirmed by Baernstein *et al* (36) with quantitative information. Richer growth of the bacterium in the amoeba cultures was shown by turbidimetric determinations which were made in the Beckman spectrophotometer. The substrate furnished by the amoebae was not detectable in medium enriched with glucose.



Figure 16 Modified Eldredge tube for collecting and measuring carbon dioxide from cultures of amoebae (From Am J Trop Med & Hyg, 1008, 1953 Courtesy of the Editor.)

The paper by Baernstein *et al* (36) shows that in cultures of the amoeba with *A aerogenes* or organism *t* the amounts of glucose disappearing from the liquid phase of the medium during forty-eight hours of incubation over and above amounts diffused from the solid phase, were insufficient to account for the carbon

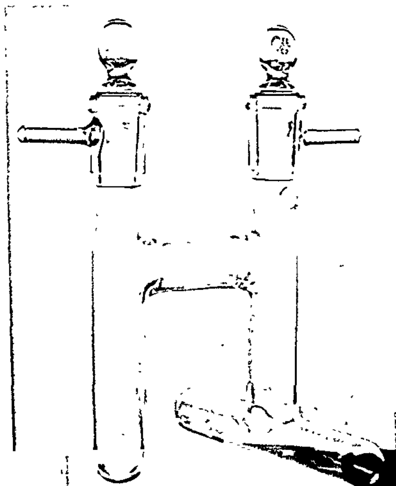


Figure 17 Eldredge tube with "Thunberg" type ground glass stoppers

dioxide produced and, therefore, that some sugar was supplied from the rice through the amylase of the amoeba Amylase was demonstrated in extracts of acetone powders of washed amoebae On the other hand, in cultures of *E histolytica-Clostridium perfringens*, no more carbon dioxide was demonstrable than

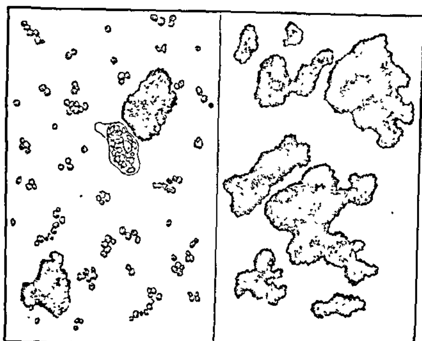


Figure 18 Fragmentation of rice flour by *Entamoeba histolytica* Left: *E histolytica*-organism *t* Right. organism *t*. (From· *Am J Trop Med. & Hyg*, 2 1009, 1953 Courtesy of the Editor)

from the bacterium without amoebae, However, the data of Table II, showing a four-fold increase in production of carbon dioxide by *C perfringens* when grown with rice over amounts without rice show that this bacterium was not dependent on amylase furnished by the amoeba. The increase in production of carbon dioxide in cultures with rice by the other three species of bacteria listed in Table II is attributable to small amounts of sugar present in the rice flour

Discussion

The limited amount of information obtainable on the metabolism of *E. histolytica* from monoxenic cultures is attributable to the limited amount of growth of the amoeba and to its slow rate of metabolism compared to that of a symbiont. In a study

TABLE II

COMPARATIVE DETERMINATIONS OF CARBON DIOXIDE FROM INDICATED SPECIES OF BACTERIA AT 72 HOURS OF INCUBATION IN MEDIUM WITH RICE AND WITHOUT RICE

	With rice flour				*Without rice flour*		
Bacterium	Five tube set of cultures	CO_2 in cc	Turbidity Log Io/I	CO_2/T	CO_2 in cc	Turbidity Log Io/I	CO_2/T
Aerobacter aerogenes	1	2.97	0.697	4.4	1.85	0.499	3.7
	2	1.96	0.452	4.0	1.61	0.450	3.4
Average		2.46	0.580	4.2	1.73	0.459	3.5
Escherichia coli	1	1.96	0.541	3.6	1.23	0.598	2.2
	2	2.02	0.493	4.1	1.65	0.409	4.5
Average		1.99	0.517	3.8	1.54	0.450	3.4
Organism t	1	2.41	0.223	10.8	1.56	0.169	9.0
	2	3.19	0.493	8.1	1.36	0.163	8.3
Average		2.80	0.358	9.4	1.46	0.166	8.7
Clostridium perfringen	1	7.11	0.561	13.0	1.74	0.449	3.9

From *Am J Trop Med and Hyg*, 2 1011 1953 (Courtesy of the Editor)

by Phillips (37) for example in medium with *T cruzi* an average of 229 amoebae per microcultures was obtained, the progeny of a single amoeba, at seventy-two hours of incubation. This figure shows that there were fewer than nine divisions, or one division every eight hours. By way of contrast certain species of bacteria divide in from fifteen to twenty minutes during the logarithmic phase of growth (Gale, 38). *E. histolytica* may not consume oxygen or yield appreciable amounts of carbon dioxide. However, continued studies using spectrophotometric measurements may eventually yield considerable information on products of glycolysis by *E. histolytica* even in monoxenic cultures or in the cultures of Shaffer and Sienkiewicz.

According to an hypothesis announced by Fildes (39) certain microbes that throughout the ages have lived in the host with bacteria have lost essential enzymes. The problem of obtaining

pure cultures of the amoeba may, therefore, lie in fields of enzyme chemistry, thus far unexplored. However, certain other protozoan organisms, including species of *Trichomonas* that live in the host with bacteria have been grown in pure culture (40).

Summary

Monoxenic cultures of *E. histolytica* with species of bacteria have been produced in whole egg medium containing rice flour, *without serum, and in egg white medium with symbionts of the coli-aerogenes* group of bacteria which are known to produce B vitamins.

Some successes have also been obtained in growing the amoeba in wholly liquid media, particularly with species of bacteria that are inhibited by antibiotics. Bacteria-free cultures have also been obtained with *Trypanosoma cruzi* and in medium prepared from minced chick embryos

E histolytica is probably an anaerobe, it grows at low levels of oxygen potential, and encysts and excysts at higher levels. Excystation is favored by the presence of glucose, B vitamins, vitamin C, and certain other compounds. Combinations of these ingredients have failed to promote axenic growth of the amoeba.

The amoeba liberates enzymes, probably protease and amylase, *that liquefy gelatin, and fragment particles of rice flour* with liberation of starch grains. It ingests and probably utilizes the starch in metabolism. The relationship between the amoeba and *a species of bacterium is one of symbiosis*

Bibliography

1 [illegible] Propagation of a strain of *Endamoeba* [illegible] *Science*, 116 306-308, 1952.

2 [illegible] WASHINGTON, J E., The propagation of *Endamoeba histolytica* in tissue-bearing culture without accompanying bacteria or other microorganisms *Am J Hyg*, 57.366-379, 1953

3 CHINN, B D, JACOBS, L, REARDON, L V, and REES, C. W.: The influence of the bacterial flora on the cultivation of *Endamoeba histolytica*. *Am J Trop Med*, 22 137-146, 1942.

4 CLEVELAND, L. R. and SANDERS, E. P., The production of bacteria-free amoebic abscess in the liver of cats and observations on the amoebae in various media with and without bacteria *Science*, 72 149-151, 1930

5 Jacobs, L. The substitution of bacteria in cultures of *Endamoeba histolytica* *J Parasitol*, 36 128-130, 1950.

6 Rees, C. W., Baernstein, H. D., Reardon, L. V. and Phillips, L. Some interactions *in vitro* of *Endamoeba histolytica* and single species of microbial symbionts *Am J Trop Med & Hyg*, 2 1002-1014, 1953

7 Rees, C. W., Bozicevich, J., Reardon, L. V. and Daft, F. S. The influence of cholesterol and certain vitamins on the growth of *Endamoeba histolytica* with a single species of bacteria *Am J Trop Med*, 24 189-193, 1944

8 Rees, C. W. and Reardon, L. V. Comparative yields of *Endamoeba histolytica*-organism t from soluble and insoluble ingredients of egg white in freshly prepared and stored medium. *Am J Trop Med*, 25 109-114, 1945.

9 Rees, C. W. Le metabolisme d *Entamoeba hystolitica* et ses relations avec les problèmes de l'amibiase clinique *Gaz Med France*, Numero Spec 57 835-841, 1950

10 Snyder, T. L. and Meleney, H. E. Anaerobiosis and cholesterol as growth requirements of *Endamoeba histolytica* *J Parasitol*, 29 278-284, 1943

11 Jacobs, L. The elimination of viable bacteria from cultures of *Endamoeba histolytica* and the subsequent maintenance of such cultures. *Am J Hyg*, 46 172-176, 1947

12 Rees, C. W. and Reardon, L. V. Studies on the growth requirements of metacystic *Endamoeba histolytica* *J Parasitol*, 33 Suppl 21, 1947

13 Hansen, E. L. A liquid medium composed of dehydrated ingredients for culture of *Endamoeba histolytica* associated with a single bacterium *J Lab & Clin Med*, 35 308-312, 1950

14 Hansen, E. L. and Anderson, H. H. An essentially synthetic liquid medium for *Entamoeba histolytica* *Parasitology*, 39 69-72, 1948

15 Hallman, F. A., Michaelson, J. B. and DeLamater, J. N. The cultivation of *Endamoeba histolytica* in a defined medium *Am J Trop Med*, 30 363-369, 1950

16. Shaffer, J. G. and Frye, W. W. Studies on the growth requirements of *Endamoeba histolytica* I Maintenance of a strain of *E histolytica* through one hundred transplants in the absence of an actively multiplying bacterial flora *Am J Hyg* 47 214 221, 1948

17 Shaffer, J. G., Ryden, F. W. and Frye, W. W. Studies on the growth requirements of *E histolytica* IV Further observations on the cultivation of *E histolytica* and other intestinal protozoa in a clear medium without demonstrable bacterial multiplication Some modifications and simplifications of the medium *Am J Hyg*, 49 127-133, 1949

18 Sands, E. H., Kruer, I. M. and Everitt, M. G. Cultivation of *Endamoeba histolytica* in embryonic fluids *Proc Soc Exper Biol & Med* 80 272 275, 1952

19 Phillips, B. P. Cultivation of *Endamoeba histolytica* with *Trypanosoma cruzi* Science, 111 8-9 1950

20 Phillips, B. P., Wolfe, P. A., Rees, C. W., Gordon, H. A., Wright, W. H. and Reyniers, J. A. Studies on the amoeba bacteria relationship in amoebiasis Comparative results of the intracecal inoculation of germ free, monocontaminated and conventional guinea pigs with *Endamoeba histolytica* *Am. J Trop Med & Hyg*, (In press)

21. Phillips, B P. and Rees, C W., The growth of *Endamoeba histolytica* with live and heat-treated *Trypanosoma cruzi* Am J. Trop Med, 30 185-191, 1950

22 Phillips, B P Comparative effects of certain species of Trypanosomidae on the growth of *Endamoeba histolytica* in the absence of bacteria Am J. Trop Med, 31 290-294, 1951

23 Rees, C W Observations of *Endamoeba histolytica* in microcultures of *Trichomonas foetus* J Parasitol, 35 Suppl, 17, 1949

24 Phillips, B P The effects of various treatment procedures on the metabolism of *Trypanosoma cruzi* and its ability to support growth of *Endamoeba histolytica* Am J Trop Med & Hyg, 2 47-53, 1953.

25 Tobie, E J. and Rees, C. W. The cultivation of *Trypanosoma cruzi* in dialysate medium J Parasitol, 34 162-163, 1948.

26 Rees, C W, Reardon, L V and Bartgis, I. L: The excystation of *Entamoeba histolytica* without bacteria in microcultures. Parasitology, 40 338-342, 1950

27. Rees, C W, Reardon, L V. and Baernstein, H D.: A biochemical approach to the problem of cultivating *Endamoeba histolytica* without bacteria J Parasitol, 34 Suppl, 11-12, 1948.

28 L

29 vc

1943

30 von Brand, T, Rees, C W, Reardon, L. V and Simpson, W F · Chemical studies on egg-white medium for the cultivation of *Endamoeba histolytica* J Parasitol, 32 190-196, 1946

31. Ci

32 Br

41, 1950

33 Jacobs, L Oxidation-reduction potentials in the cultivation of *Endamoeba histolytica* Am J Trop Med, 30 803-815, 1950

34 Soc.

35. tivity 1953

36 Baernstein, H D, Rees, C. W and Reardon, L V.: Symbiosis in cultures of *Endamoeba histolytica* and single species of bacteria Am J Trop Med & Hyg, 3 839-848, 1954

37. Phillips, B P. Measurements of direct amebacidal potential by a micro-method for the screening of drugs *in vitro* Am J. Trop Med, 31 561-565, 1951.

38 Gale, E F · The Chemical Activities of Bacteria New York, Academic Press, Inc, 213 pp, 1951.

39 FILDES, P Some medical and other aspects of bacterial chemistry *Proc Roy Soc Med*, 28 79 90, 1934
40 TRUSSELL, R E and PLASS, E D The pathogenicity and physiology of a pure culture of *Trichomonas vaginalis* *Am J Obst & Gynec*, 40 883-890, 1940

21 PHILLIPS, B. P and REES, C. W.: The growth of *Endamoeba histolytica* with live and heat-treated *Trypanosoma cruzi*. *Am. J. Trop Med*, *30* 185-191, 1950

22 PHILLIPS, B P. Comparative effects of certain species of Trypanosomidae on the growth of *Endamoeba histolytica* in the absence of bacteria *Am J Trop Med.*, *31* 290-294, 1951

23. REES, C. W Observations of *Endamoeba histolytica* in microcultures of *Trichomonas foetus* *J Parasitol*, *35* Suppl, 17, 1949

24 PHILLIPS, B. P. The effects of various treatment procedures on the metabolism of *Trypanosoma cruzi* and its ability to support growth of *Endamoeba histolytica* *Am J Trop Med. & Hyg*, *2* 47-53, 1953

25 TOBIE, E J. and REES, C W.. The cultivation of *Trypanosoma cruzi* in dialysate medium *J Parasitol*, *34* 162-163, 1948

26 REES, C W, REARDON, L V and BARTGIS, I L · The excystation of *Entamoeba histolytica* without bacteria in microcultures *Parasitology*, *40* 338-342, 1950

27 REES, C W, REARDON, L V. and BAERNSTEIN, H D A biochemical approach to the problem of cultivating *Endamoeba histolytica* without bacteria. *J Parasitol*, *34* Suppl, 11-12, 1948.

28 L[illegible]

29 [illegible]
reducing substances and gas formation in cultures of *Endamoeba histolytica* and a single species of symbiotic bacteria *Am J. Trop Med*, *37* 310-319, 1943

30 VON BRAND, T, REES, C. W, REARDON, L V and SIMPSON, W F: Chemical studies on egg-white medium for the cultivation of *Endamoeba histolytica* *J Parasitol*, *32* 190-196, 1946

31 CHANG, S L Studies on *Entamoeba histolytica* IV The relation of oxidation-[illegible]

32 BE[illegible]
41, 1950.

33 JACOBS, L Oxidation-reduction potentials in the cultivation of *Endamoeba histolytica* *Am J Trop Med*, *30* 803-815, 1950.

34 [illegible] Soc

35 [illegible] tivity 1953

36 BAERNSTEIN, H D, REES, C. W and REARDON, L. V.: Symbiosis in cultures of *Endamoeba histolytica* and single species of bacteria *Am J Trop Med & Hyg*, 3 839-848, 1954

37 PHILLIPS, B P. Measurements of direct amebacidal potential by a micromethod for the screening of drugs *in vitro* *Am. J Trop Med*, 31 581-585, 1951

38 GALE, E F The Chemical Activities of Bacteria New York, Academic Press, Inc, 213 pp, 1951.

39 FILDES, P Some medical and other aspects of bacterial chemistry *Proc Roy Soc Med*, 28 79-90, 1934

40 TRUSSELL, R E and PLASS, E D The pathogenicity and physiology of a pure culture of *Trichomonas vaginalis* *Am J Obst & Gynec*, 40 883-890, 1940

CHAPTER 6

EXPERIMENTAL CHEMOTHERA[PY] OF AMOEBIASIS

Introduction

PROBABLY one of the reasons why amoebic dysentery is n
the dreaded disease that it was during the fourth quarter
past, and the early years of the present century, is that c
drugs have been demonstrated more or less effective in ame
ting the symptoms even if not in eradicating the parasite fron
intestinal tract. The first case reported by Loesch (1) in 1
as well as the cases studied by Councilman and LaFleur (2
1891, and Amberg (3), in 1901, were treated with quinine,
only known antiprotozoal agent at those periods. Councilm
and LaFleur, in some cases, administered ipecac. However, fro
1909 to 1926 Simon (4), Dock (5), Vedder (6), Rogers (7
DuMez (8), and Willmore and Martindale (9) demonstrate
the effectiveness of emetine, particularly against amoebic absces
of the liver. This drug is an alkaloid of ipecac or ipecacuanha
from the roots of *Cephaelis* or *Psychotria ipecacuanha*, a plant
that is native in Brazil, and cultivated in India, the Straits
Settlements, and elsewhere. Its trial as an amoebacide was in-
dicated on the basis of wide usage by the natives of Brazil for
cases of dysentery. Emetine hydrochloride is administered by
subcutaneous, intramuscular, or intravenous injection, and eme-
tine bismuth iodide, and auremetine by mouth. However, eme-
tine is relatively less effective against intestinal amoebiasis than
against extraintestinal amoebiasis. Except by experienced phy-
sicians, its use is contraindicated because of severe cardiac
damage.

At the present time emetine is usually administered as an
adjunct of one or the other of the following drugs: Acetarsone
(Stovarsol) introduced by Marchoux (10), treparsol by Brown

(11), carbarsone by Reed *et al* (12), chiniofon "Yatren" by Muehlens and Menk (13), vioform by Anderson and Koch (14) and diodoquin by Tenney (15) More recently certain thio-derivatives of carbarsone oxide have been used as pointed out in detail later in this chapter Chloroquin (Aralen) has recently been reported more effective than emetine in the treatment of amoebic abscess of the liver by Conan (16), Murgatroyd and Kent (17), and Manson-Bahr (18) Its principal advantage over emetine is that it may be taken by mouth without nausea and has no demonstrable effect on the heart

Perusal of the literature cited above emphasizes the need for more work in experimental chemotherapy since as was pointed out by Anderson *et al* (19) the use of so many remedies for a disease indicates that none is entirely effective. Experiments in chemotherapy should include measurements of the effects of drugs and products of their degradation on the life of the amoeba, on specific phases of its metabolism, as emphasized by Work and Work (20), and on comparative effects on the amoeba and on the host as pointed out by Hogan and Eagle (21) The need for experimental methods to replace methods of empiricism was emphasized by Wright (22). As pointed out by Findlay (23) experimental chemotherapy as opposed to methods of empiricism, is a new field of investigation This statement applies with particular force to the chemotherapy of amoebiasis as may be strikingly illustrated by reference to books dealing in part or in entirety with amoebiasis In 1926, Wenyon (24) stated that emetine hydrochloride and emetine bismuth iodide were the only drugs known to be effective in amoebiasis Recently, however, Findlay, and Anderson *et al*, listed a total of 27 agents in current use, including four preparations of emetine, seven aromatic arsenicals, three iodoquinolines, seven antibiotics, one Kurchi alkaloid, one German product (Gavano), one acridine (Rivanol), two bismuth compounds, and chloroquin Most "cures" that have been followed for considerable periods post-treatment have relapsed

Screening Drugs for Amoebacidal Effects *In Vitro*

The work by Dobell (25), and by others, has shown that emetine hydrochloride is most effective against *E. histolytica in vitro*, and for this reason it has been adopted as the standard of reference by which the effects of other drugs are measured. However, the comparative ineffectiveness of emetine in the intestinal tract shows that tests *in vitro* may not show the usefulness of a drug as a chemotherapeutic agent. As Dobell pointed out, if an *in vitro* test could be made to reveal "the exact mechanism" by which a drug kills the parasite the tests would have great value in studies on chemotherapy. The presence of one or more species of bacteria in the cultures of *E. histolytica* further complicates the problem. A compound appearing to be amoebastatic or amoebacidal may, in fact, be only bacteriostatic or bactericidal. As pointed out by Dobell in referring to crude cultures: "Every ordinarily cultivated strain of *E. histolytica* contains a mixed and largely unknown bacterial flora (on which the amoebae are directly or indirectly dependent), and unless this can be regulated and standardized, all such tests contain an element of uncertainty." The use of diphasic medium for tests *in vitro* has been criticized by Dobell, and others, on the ground that much of the drug may be absorbed on the solid phase of the medium. Many of the tests recently made have, therefore, been with wholly liquid media.

Brackett and Bliznick (26) have pointed out that in order to differentiate between the amoebastatic and the amoebacidal effects of a drug it is necessary to ascertain whether a "fold increase" occurs between the number of amoebae in the inoculum and the number produced on incubation. A most effective method of answering this question was devised by Phillips (27) with the use of our microisolation and microculture techniques. From a single trophozoite of *E. histolytica* that was isolated per microtube containing a heavy suspension of *Trypanosoma cruzi*

multiplication in glaucarubin (simaroubidin), aureomycin, ...

terramycin, in concentrations of 0.050, 0.075, and 0.200 mg respectively per cc. of the medium. Effects of penicillin, streptomycin, chloromycetin, and bacitracin on the amoebae were slight. As expected the amoebacidal effects of emetine hydrochloride were high. The trypanosome was not demonstrably affected by any of these drugs. Hrenoff and Nakamura (28) using test tube cultures of the amoeba-trypanosome studied the effects of Fumagillin. Bradin and Hansen (29) attacked the problem by using parallel tubes of medium—one set cotton-stoppered and the other sealed with petrolatum. Their data show that drugs that are primarily bacteriostatic caused death of the amoebae in the cotton-stoppered tubes but not in those in which entry of oxygen was retarded by the seal. Confirmatory evidence was obtained by potentiometric measurements. Thompson *et al.* (30) conducted tests with the use of anaerobic jars. Dobell (25) added methylene blue to his cultures with *Escherichia coli* and observed that a drug permitting oxidation of the dye was primarily bactericidal. A large number of tests with the use of crude cultures are not herein discussed for reasons already stated.

Prerequisites of a Good Amoebacide

In the foregoing account on tests of drugs *in vitro*, it was tentatively assumed that a good agent must destroy the amoebae, and that a drug affecting bacteria might have no value as an amoebacide. However, there is considerable evidence that participation of bacteria might be required for pathogenicity of the amoeba in the human host. Elsdon-Dew (31, 32, 33, 34) administered antibiotics known to be bactericidal in cases of very severe amoebic dysentery with the result that the symptoms were abated to the extent that what were considered truly amoebacidal agents could be tolerated. Based on wide experience in India and other lands, Hargreaves (35, 36) also recommended the use of antibiotics. Stewart (37) and Stewart and Jones (38) obtained evidence for participation of bacteria in cases of amoebic dysentery, particularly of organisms classed as paracolons.

Since with rare exceptions laboratory animals must be used for experiments on amoebacides *in vivo* it is pertinent here to

thus inoculated A high percentage of "conventional" guinea pigs contracted amoebic dysentery from portions of the same inoculum as used for the germ-free animals. However, as explained below, the amoeba with *T. cruzi* is pathogenic for guinea pigs only when derived in cultures from microisolated cysts shortly prior to use in the experiments.

Questions concerning the comparative virulence of strains of *E. histolytica* are also of importance. Strain 200 mentioned above has been selected by a considerable number of workers because of its reputed virulence. Earlier work by Meleney (48), and Meleney and Frye (49), has indicated the occurrence of strains of the amoeba differing greatly in virulence. Methods discussed in Chapter 4 whereby strains of *E. histolytica* from human subjects may be isolated at will in monoxenic cultures are of importance in problems of virulence. The above-mentioned experiments from Dr. Meleney's laboratory would have far greater significance if complicating factors inherent in the occurrence of a number of species of bacteria in the cultures had been more effectively evaluated. Some important data concerning virulence of E *histolytica* was obtained recently by Phillips and Bartgis (39). They investigated the effects in guinea pigs of strain 200, *E. histolytica-T. cruzi*, when recently established with the trypanosome from microisolated cysts, compared with effects of amoebae from the crude cultures from which the cysts were obtained Intracaecal injection of trophozoites from the cultures with *T. cruzi* produced fatal amoebic dysentery in from 80 to 90 percent of the guinea pigs, the results comparing favorably with those from crude cultures However, on serial transfer for periods as short as twelve weeks, the amoeba-trypanosome cultures became practically avirulent. Previous work by Deschiens (50, 51, 52) showed that a strain of the amoeba that was highly virulent for kittens gradually lost virulence on serial transfer in medium without rice starch where encystation was not demonstrable. On the other hand a series of the strain that was maintained in medium enriched with the starch retained its virulence during serial transfer for periods up to six years. In experimental work the possible bearing of the data concerning

virulence on the effects of chemotherapeutic agents is difficult to evaluate Available evidence indicates that infection in man is acquired only from ingestion of cysts

Selection of the Experimental Host

The susceptibility of dogs to *E histolytica* was demonstrated by Loesch (1) and of the kitten by Kartulis (53) Later work by Dobell (54) showed that the macaque is a natural host and although dogs and cats may acquire the infection in poorly sanitated areas, the ape is the only known natural host besides man Early attempts to infect rats, rabbits, and guinea pigs were seldom successful but recently in the investigations of Goodwin *et al.* (55) and Jones (56, 57, 58) the rat has been found susceptible to short-term infection More recently through leads furnished by Westphal (59), Tobie (60) produced fulminating dysentery in rabbits by feeding cysts, and more particularly, by injecting trophozoites into the caecum Carrera and Faust (61), Solier and Peleux (62), and Taylor *et al* (63) produced amoebic dysentery in guinea pigs by intracaecal injection of trophozoites An upsurge of interest in experimental chemotherapy of amoebiasis has resulted from the above investigations on rodents

Factors of importance in the selection of the experimental host are Cost of the animal, cost of maintenance, numbers available at all seasons of the year, and space and equipment required for housing On these grounds the rat, rabbit, and guinea pig have advantages over dogs, kittens, or monkeys The evanescent character of the infection in the rat weighs heavily against selection of this animal Information obtained from rabbits or guinea pigs will require follow-up experiments in macaques and/or man because infection of the caecum may differ in fundamental respects from infection of the colon and the rectum Considerable difficulty may be experienced in ascertaining the onset of the prepatent period of rodent hosts because amoebae may be multiplying in the caecum and the upper colon without being demonstrable in the fecal pellets The dog is more adaptable than the kitten to conditions in the laboratory, and as indi-

cated later, this host may furnish valuable information on chemotherapy A large proportion of macaques are already infected with *E histolytica* when purchased and seldom respond clinically to experimental inoculation of the amoeba from human cases.

For long term projects it is advisable to give more consideration than is usually the case to breeding, housing, and maintaining the animals. At the Laboatory of Bacteriology of Notre Dame University (64), a house for animals has been constructed with safeguards against infestation with ectoparasites, infection with intestinal protozoa or even with agents causing pneumonia and/or tuberculosis The building is surrounded by a tar-filled moat, has only one entrance for food and bedding, where provision is made for treatment with ethylene chloride at a pressure of twenty pounds per square inch, and another entrance for the animal attendant. The attendant enters an anteroom with a DDT spray where he also removes his clothing and puts on sterile clothing When measured against loss of time and money from the use of animals infested with parasites, the cost of such housing is not prohibitive.

Chemotherapeutic Investigations on Designated Hosts

Kittens

Dale and Dobell (65) attempted without success to demonstrate effectiveness of preparations of emetine and several other drugs in experimental amoebiasis in kittens The life of the host was shortened rather than prolonged. In recent experiments, Clampit (66) selected kittens around 600 gm. but made an exception in one cat weighing 900 grams. This cat had negative stools following treatment with carbarsone but passed amoeba at later periods The same results followed treatment with diodoquin, and with vioform. Finally when the cat attained a weight of 2 kilograms, it recovered spontaneously. Quite a number of other experiments might be cited to show the nonsuitability of the kitten for experimental chemotherapy. In many cases the kittens have died of coccidiosis, pneumonia, or other infections before the drugs could be tested against the amoeba

Dogs

Thompson and Lilligren (67) chose the dog for some of their experiments because without treatment this host retains the infection indefinitely when given the salmon diet originally recommended by Faust *et al* (68). Mackerel was found quite as effective as salmon. Drugs were administered at three dose-levels of one-fourth to four times the amounts mg/kg recommended for man by *Newer and Nonofficial Remedies* The highest of the above dosages of diodoquin and carbarsone respectively was required, but the level of chiniofon recommended for man was effective for clearance from amoeba The substituted quinoline (amodiaquin), several sulfa drugs, and a number of antibiotics including penicillin and streptomycin had no demonstrable effects on the amoebae It appears that the dog may furnish valuable information on the chemotherapy of amoebiasis Further tests of certain antibiotics such as fumagillin would be of interest

Macaques

The choice of the macaque for experimental chemotherapy of amoebiasis has long been advocated by Dr Hamilton Anderson who has made significant contributions toward the chemotherapy of the disease He regards amoebiasis in the Western Hemisphere as a chronic, recurrent, and not infrequently a drug-refractive disease, that may remain dormant for years and thus frequently not diagnosed However, in one series of 200 cases of Drs T T Perry and W T Gibb that were studied at our laboratory, eighty of whom had arthritis in addition to intestinal complaints, there were only seven infections with *E histolytica* ascertainable by fecal diagnosis, although a considerable number had positive complement fixations

Recent papers by Anderson and associates (69, 70, 71) on certain thioderivatives of carbarsone oxide, especially the compounds labelled C C.914, and C C 1037, showed that the thioderivatives were efficacious against the amoeba and less damaging to the host than carbarsone Comparative toxicities were measured in mice, rats, and rabbits, and the amoebacidal effects determined in macaques The studies included brom-sulphalein

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tests of liver function and phenolsulfonphthalein tests of renal function, also analysis of tissues for arsenic. Anderson and Anderson (72) reported that certain antibiotics may *temporarily* clear a high percentage of macaques but require "follow up" treatment with truly amoebacidal drugs. A mixture of bacitracin, polymyxin, and streptomycin, gave prompt cessation of symptoms, prompt gain in weight, and temporary clearance of amoebae. Hrenoff (73) reported temporary clearance of five out of nine macaques from administration of terramycin. Anderson *et al* (74) *found fumagillin very effective as an amoebacide in* macaques.

The work reported above on macaques as well as earlier studies by Reed *et al.* (12) and Leake (75) which led to the use of carbarsone in human amoebiasis provide ample demonstrations of the usefulness of the macaque as a host for experimental chemotherapy of amoebiasis, especially for tests on toxicity prior to the use of drugs in patients. However, the objections mentioned above to the macaque mitigate against the use of this host for preliminary chemotherapeutic experiments Except in countries where such primates are plentiful only a limited number of preliminary trials on potential amoebacides may be conducted.

Rats

In preliminary papers, Jones (56, 57, 58) *reported effectiveness* of chiniofon, stovarsol, and diodoquin, in this order, against amoebic infections in baby rats but only at tremendously high dose-levels compared with those tolerated by human subjects. Because of spontaneous recoveries in the controls, it was necessary to use a statistical formula to indicate significance. Goodwin, *et al* (55) using baby rats, employed a formula for the chemotherapeutic index (maximum *tolerated dose* × *0 1/minimum* tolerated dose × 99 9) as proposed by Trevan (76), and compared the results with tests *in vitro*. *In vivo* the order of effectiveness of drugs was carbarsone, chiniofon, acetarsone, emetine HCl, emetine bismuth iodide, and diodoquin. *In vitro* it was emetine HCl, chinofon, carbarsone, diodoquin, and acetarsone. It is obvious that tests *in vitro* bore little relationship to those in the rat.

Beginning with a compound that according to Brindley and Pyman (77) has the same formula as emetine, Goodson *et al* (78, 79) synthesized a total of 132 compounds and tested them against amoebic infection in baby rats Their objective was to find a compound with high effectiveness, low toxicity, and suitable for oral administration They reported little success Fulton *et al* (80) tested a large number of compounds *in vitro* from Imperial Pharmaceuticals Ltd and from I G Farbenindustrie Their data are of interest here because of their statements concerning rats: "Rats are naturally infected with *E muris* which complicates diagnosis, and the use of newly weaned rats for intracaecal infections from culture material gives a high mortality frequently associated with bacteremia." A compound a-p-chloranulino-4-diethyl-amino quinoline, and the German drug Gavano were recommended for further trials An interesting report on German products by Schoenhoefer (81) is that because of scarcity of iodine in World War II, chlorine was substituted in Yatren (chiniofon) The most significant data on rats were obtained by Neal (82) since his rats remained infected with amoebae for as long as 315 days. Higher dosages of drugs were required to clear rats that had been infected with *E histolytica* from clinical cases of the disease than from "carrier" cases Dosages totaling 6 to 12 mg/kg of chiniofon cleared the latter while dosages of 16 mg/kg were required for the former.

Rabbits and guinea pigs

During the past several years the chemotherapy of amoebic dysentery in rabbits and guinea pigs has been investigated at the National Microbiological Institute. Luttermoser *et al.* (83) found that although diodoquin, chiniofon, vioform, and terramycin, in doses mg/kg tolerated by man, failed to clear rabbits of the infection, these drugs were effective at higher dose-levels In preliminary investigations of fifty drugs, Taylor and Greenberg (84) reported that compounds of arsenic and iodine were effective in guinea pigs below toxic levels However, glaucarubin (Simaroubidin) and fumagillin gave better results than any of the other drugs The results on fumagillin are in confirmation of those of McCowen *et al.* (85)

Distribution of Drugs in the Animal Body

As already noted, Hogan and Eagle (21) have stressed the need of ascertaining the fate of pharmaceutical agents in the body as well as their effects on the parasite Pertinent literature was cited in their paper as well as by Banks (86) on the classical investigations of Erhlich and collaborators. The studies on some thioderivatives of carbarsone oxide by Anderson *et al.* (69, 70, 71) have been mentioned. Haskins *et al.* (87) and Haskins and Luttermoser (88) investigated the distribution of certain iodoquinolines containing radioactive iodine. The curves of blood levels in rabbits following oral administration were high for chiniofon, and vioform, and low for diodoquin. In the feces the highest levels were found for diodoquin, probably because of its slight solubility in the intestinal contents. Vioform and chiniofon were eliminated to a far greater extent in the urine than in the feces. The highest levels of all three drugs was in the thyroid gland They concluded that blood levels were not high enough in any case for effectiveness against extraintestinal amoebiasis. They developed a spectrophotometric method of analyzing compounds within the tissues of the host. In Germany, Palm (89) also used biochemical methods Parmer (90) analyzed the tissues of rabbits following treatment with emetine and found high levels in the liver and low levels in the intestinal tissues. Anderson and Leake (91) demonstrated cardiac damage in rabbits and cats due to emetine. Albright *et al.* (92) and Knight and Miller (93) used human volunteers for analysis of radioactive compounds. Although considerable disagreement occurs among investigators concerning distribution of drugs and products of their degradation in the body, the differences as pointed out by Haskins and Luttermoser are probably attributable to differences in techniques. As already suggested, knowledge of the manner in which a drug affects the host and is distributed with or without degradation in the body is prerequisite for comparative evaluation of amoebacidal drugs against amoebiasis in the experimental host as well as in man

Experimental Chemotherapy of Amoebic Liver Abscess

Recent studies by Rees *et al.* (94) showed that *E histolytica* was demonstrable in the liver in high percentages of guinea pigs that were killed *in extremis* with amoebic dysentery, although evidence of liver damage was not obtained Amoebic hepatitis may occur in many cases commonly regarded as only intestinal amoebiasis, and drugs acting systemically may be required in all cases of the disease Failure of certain drugs to eradicate the parasite from the host may even be due to reinfection of the intestinal tract from the liver. In the search for a host in which amoebic abscess of the liver may be produced in a considerable number of cases following injection of trophozoites into the organ, Reinertson and Thompson (95) and Thompson and Reinertson (96) have obtained encouraging leads from the hamster Liver abscesses have occurred in 90 per cent of the animals although the lesions were not free from bacteria and disappeared spontaneously within four to five days However, with the use of a statistical formula based on the square root of the weight of the abscess, they demonstrated significant chemotherapeutic effects of emetine injected intramuscularly and of chloroquin, quinacrine, and amodiaquin administered orally. Carbarsone, chloromycetin, aureomycin, penicillin, and dihydrostreptomycin were without effects on the abscesses A beginning has been made toward effective chemotherapy of liver abscess

Discussion

The foregoing account shows that although considerable progress has been made toward the conquest of amoebiasis with chemotherapeutic agents, there are opportunities for much more work It is also apparent that the fields of protozoology, bacteriology, biochemistry, physics, and medical practice must be drawn upon for interpretation of experimental results In the present state of our knowledge of the disease, or lack thereof, reliance must be placed largely on methods of empiricism However, more precise methods of approach along lines begun by Goodson *et al* (78, 79), by Fulton (80), and by Anderson (69

70, 71, 72) and their respective associates involving clinical trial of compounds closely related chemically to agents known to be *somewhat effective should bear fruitful results.* The principal complications resulting from the occurrence of species of bacteria in cultures used for tests *in vitro* and for inoculation into experimental animals may be eliminated by methods of producing cultures of the amoeba with single species of bacteria or in association with either *Trypanosoma cruzi* or embryonic metazoan cells. Inoculation *per os* with bacteria-free cysts is also of importance. The complications attributable to organisms occurring in the intestinal flora of the host are far more difficult to evaluate There is reason for optimism, however, that production of *germ-free experimental hosts may eventually be attained on* a practicable basis In the light of progress that has already been made in the face of many difficult situations, the outlook is good for more rapid progress in the future.

Summary

Considerable progress has been made toward the conquest of amoebiasis by methods of chemotherapy. The pioneer workers used quinine without any demonstrable effects. Most of the patients with clinical amoebiasis died despite the use of this drug and the benefits of hospitalization Emetine and other derivatives of ipecac, introduced early in the present century, produced many dramatic results on amoebic abscess in the liver but have been less effective against intestinal amoebiasis and cause damage to cardiac musculature. A number of aromatic compounds of arsenic and a number of the iodoquinolines are very useful in cases of intestinal amoebiasis. Remarkable effects on the symptomatology of amoebiasis have been produced by certain antibiotics, but their effects are probably attributable to action against bacteria and follow-up treatment with amoebacidal drugs is usually necessary. No regimen of treatment thus far found has eradicated the amoeba in many cases of infection

With the use of monoxenic cultures of *E. histolytica*, in wholly liquid medium, under properly controlled experiments, some *pertinent information* concerning amoebacides may be obtained.

But effectiveness of a drug *in vitro* may or may not indicate effectiveness *in vivo*, and drugs relatively ineffective *in vitro* may be effective *in vivo*.

In early work the investigations in experimental chemotherapy of amoebiasis were hampered by lack of suitable hosts, but recent demonstrations of pathogenesis of the amoeba in rats, rabbits, guinea pigs (particularly germ-free guinea pigs), and hamsters have led to an upsurge of interest in experimental chemotherapy.

The use of kittens has produced very little information on the chemotherapy of amoebiasis. The dog is a comparatively much better host. The macaque being physiologically more closely related to man than the other hosts is a valuable animal in which to test drugs prior to their use in patients. The principal disadvantage in the use of macaques is their parasitism with *E histolytica* without symptoms, and their failure to respond clinically to experimental infection with *E histolytica*.

A beginning has been made toward tracing drugs through the body of the host for evidence of their distribution and degradation. A rich field is open for future investigators.

A beginning has also been made in the production and treatment of experimental liver abscess in hamsters and hepatitis in the guinea pig.

Bibliography

1 LOESCH, F Massenhafte Entwickelung von Amoben im Dickdarm *Arch Path Anat*, 65 196-211, 1875
2 COUNCILMAN, W T and LAFLEUR, H A Amoebic dysentery *Johns Hopkins Hosp Repts*, 2 395-548, 1891
3 AMBERG, S A contribution to the study of amoebic dysentery in children, *Bull Johns Hopkins Hosp* 12 355-363, 1901
4 SIMON, S K Amebic dysentery *J A M A*, 53 1526-1532, 1909
5 DOCK, G A note on the ipecac treatment of amoebic dysentery *New York Med J* 90 49-50, 1909
6 VEDDER, E B A preliminary account of some experiments undertaken to test efficacy of ipecac treatment of dysentery *Bull Manila Med Soc*, 3 48-53, 1911
7 ROGERS, L Further experience of the specific curative action in amoebic disease of hypodermic injections of soluble salts of emetine *Brit Med J*, 2 405-408, 1912

8 DeMez, A G. Two compounds of emetine which may be of service in the treatment of entamoebiasis *Philippine J Sc*, 10 73-79, 1915.

9 Willmore, J G and Martindale, W. H.. The treatment of amoebic dysentery by auremetine *Brit M J*, (3403), 525-527, 1926

10 Marchoux, E Le stovarsol guerit rapidement la dysenterie amibienne. *Bull. Soc Path exot*, 16 79-81, 1923

11 Brown, P W Certain aspects of endamebiasis. *M Clin. North America*, 11 1419-1454, 1928

12 Reed, A C Anderson, H H, David, N. A. and Leake, C. D.. Carbarsone in the treatment of amebiasis. *J A M A*, 98 189-194, 1932

13 Mühlens, P and Menk, W Ueber Behandlungsversuche der chronischen Amoebenruhr mit Yatren *Munchen Med Wchnschr*, 68 802-803, 1921.

14 Anderson, H H and Koch, D Iodochloroxyquinoline (Vioform N N R.) as an amebacide in macaques *Proc. Soc. Exper Biol & Med*, 28 838-841, 1931

15 Tenney A C The present concept of *Entamoeba histolytica* infestation *Illinois M J*, 70 145-148, 1936.

16 Conan, N J, Jr Chloroquine in amebiasis *Am J Trop Med*, 28 107-110, 1948

17 Murgatroyd, F and Kent, R P Refractory amoebic liver abscess treated with chloroquine *Tr Roy Soc*, 4 15-16, 1948

18 Manson-Bahr, P The treatment of amoebic liver abscess with chloroquine *J Trop Med & Hyg*, 52 91-93, 1949

19 Anderson H H, Bostick, W L and Johnstone, H G. *Amebiasis Pathology, Diagnosis, and Chemotherapy* Springfield, Illinois, Charles C Thomas, 1953, 431 pp

20 Work, T S and Work, E *The Basis of Chemotherapy* New York, Interscience Publishers Inc, 1948, 435 pp

21 Hogan R B and Eagle, H The pharmacologic basis for the widely varying toxicity of arsenicals *J Pharmacol & Exper Therap*, 80 93-113, 1944.

22 Wright W H Past developments and present needs in the chemotherapy of parasitic diseases, In *Advancing Fronts in Chemistry* Vol 2, Chemotherapy New York, Reinhold Publishing Corporation, 1946, 150 pp

23 Findlay, G M Chemotherapy of amoebiasis (*In Recent Advances in Chemotherapy*, V 1, 3rd Edition) Philadelphia, Blakiston Company, 1950, 625 pp

24 Wenyon C M *Protozoology A manual for medical men, veterinarians and zoologists* I & II New York, William Wood and Company, 1926, 1563 pp

25 Dobell, C An improved method for testing the action of emetine and other chemicals on *Entamoeba histolytica* in cultures *Ann Soc Belge Med Trop*, (Liber Jubilaris J Rodhain) 201-211, 1947.

26 Brackett, S and Bliznick, A The rate of multiplication of *Entamoeba histolytica* and its relation to *in vitro* drug testing and possibly to nutritional studies *J Parasitol*, 33 154-166, 1947.

Phillips B P Measurements of direct amebicidal potential by a micromethod for screening of drugs *in vitro* *Am J Trop Med*, 31 561-565, 1951

Hrenoff, A K and Nakamura, M: *In vitro and in vivo* studies of a new antibiotic Fumagillin, with *Endamoeba histolytica*. *Proc Soc. Exper Biol & Med*, 77 162-164, 1951

29 BRADEN, J L JR and HANSEN, E. L Indirect *in vitro* action of antibiotics in comparison with activity of accepted amebacides *Am J Trop. Med*, 30.27-41, 1950.

30 THOMPSON, P E, DUNN, M C, BAYLES, A, and REINERTSON, J W · Action of chloramphenicol (chloromycetin) and other drugs against *Endamoeba histolytica in vitro* and in experimental animals *Am J Trop Med*, 30 203-215, 1950

31 ELSDON-DEW, R Endemic fulminating amebic dysentery *Am J Trop. Med*, 29 337-340, 1949

32 ELSDON-DEW, R and FREEDMAN, L Intestinal parasites in the Natal Bantu *South African J Clin Sc*, 3 59-65, 1952

33 ELSDON-DEW, R, HORNER, R, and CAMERON, D A Some aspects of amoebiasis in Africans *South African M J*, 20 580-587, 620-626, 1946

34 ELSDON-DEW, R Amoebiasis A problem *South African J Clin Sc*, 48 187-188, 1952

35 HARGREAVES, W H The treatment of amoebiasis with special reference to chronic amoebic dysentery *Quart J Med*, 15 1-23, 1946

36 HARGREAVES, W H Recent advances in the treatment of amoebiasis *J Roy Army Med Corps*, 96 41-42, 1951

37 STEWART, G T The role of bacteria in intestinal amoebiasis in man *Ann Trop Med & Parasitol*, 42 198-206, 1948

38 STEWART, G T and JONES, W R Studies in the pathology of experimental amoebiasis *Tr Roy Soc Trop Med & Hyg*, 41 438-439, 1948

39 PHILLIPS B P and BARTGIS, I L Effects of growth *in vitro* with selected microbial associates, and of encystation and excystation, on the virulence of *Endamoeba histolytica* for guinea pigs *Am J Trop Med & Hyg*, 3 621-627, 1954

40 LUTTERMOSER, G W and PHILLIPS, B P Some effects of cultural associates on the infectivity of a strain of *Endamoeba histolytica* for the rabbit *Am J Trop Med & Hyg*, 1 731-735, 1952

41 HEGNER, R W The relations between carnivorous diet and mammalian infections with intestinal protozoa *Am J Hyg*, 4 393-400, 1924

42 RATCLIFFE, H L The numbers of trichomonads in rats on diets of different protein content in relation to the pH and bacteria in the caecum *Am J Hyg* 8 910-931 1928

43 TAYLOR D J and GREENBERG, J Effects of dietary carbohydrate on amebic infections in the guinea pig *Federation Proc*, 10 398, 1951

44 TAYLOR, D J GREENBERG, J and JOSEPHSON, E S The effect of two different diets on experimental amebiasis in the guinea pig and in the rat *Am J Trop Med & Hyg*, 1 559-566, 1952.

45 SADUN, E H, BRADIN, J L JR and FAUST E C The effect of ascorbic acid deficiency on the resistance of guinea-pigs to infection with *Endamoeba histolytica* of human origin *Am J Trop Med*, 31 426-437, 1951

46 CARRERA, G M, SADUN, E H and FAUST, E C The effect of protein deficient diets on the susceptibility of guinea pigs to infection with *Endamoeba histolytica* *Am J Trop Med & Hyg*, 1 960-966, 1952

47 Phillips, B P, Wolfe, P A, Rees, C W, Gordon, H A, Wright, W H and Reyniers, J A Studies on the amoeba-bacteria relationship in amoebiasis Comparative results of the intracecal inoculation of germ-free, monocontaminated, and conventional guinea pigs with *Endamoeba histolytica* *Am J Med & Hyg*, in press

48 Meleney, H E The relationship of clinical amebiasis to various strains and growth requirements of *Endamoeba histolytica* *Puerto Rico J Pub Health & Trop Med* 59-70, 1944

49 Meleney, H E and Frye, W W The pathogenicity of four strains of *Endamoeba histolytica* from Chicago *Am J Digest Dis & Nutrition*, 4 37-40 1937

50 Deschiens R Considérations sur l'action pathogène d'une souche d'amibes dysentériques (Souche C Dobell D K B) *Bull Soc Path exot*, 30 562-564 1937

51 Deschiens R Nouvelles données sur le rôle de la flore associée à l'amibe dysentérique dans l'amibiase expérimentale *Compt rend Soc biol*, 127 1076 1079, 1938

52 Deschiens R La biologie de l'amibe dysentérique dans ses relations avec l'Amibiase *Gaz Med France* (Numero Spec), 57 829-632, 1950

53 Kartulis S Einiges über die Pathogenese der Dysenterieamöben *Centralbl Bakteriol* 9 365-372, 1891

54 Dobell C Researches on the intestinal protozoa of monkeys and man IV An experimental study of the *histolytica*-like species of *Entamoeba* living naturally in macaques *Parasitology*, 23 1-72, 1931

55 Goodwin L G, Hoare C A and Sharp, T M The chemotherapy of amoebiasis I Introduction and methods of biological assay *Brit J Pharm & Chemotherap*, 3 44-48 1948

56 Jones W R The experimental infection of rats with *Entamoeba histolytica*, with a method for evaluating the anti-amoebic properties of new compounds *Ann Trop Med & Parasitol*, 40 130-140, 1946

57 Jones W R The therapeutic action of some known amoebicides in rats *Brit J Pharm & Chemotherap*, 2 217-220, 1947

58 Jones W R The experimental chemotherapy of amoebiasis *Proc 4th Internat Cong Trop Med & Malaria*, 2 1088-1099, 1948

59 Westphal A Experimentelle Amöbenruhr beim Kaninchen *Deutsche Tropenmed Ztschr* 45 653-657, 1941

60 Tobie J E Experimental infection of the rabbit with *Endamoeba histolytica* *Am J Trop Med* 29 859-870, 1949

61 Carrera G M and Faust, E C Susceptibility of the guinea pig to *Endamoeba histolytica* of human origin *Am J Trop Med*, 29 647-667, 1949

62 Sohier R and Peloux, Y Amibiase expérimentale du cobaye *Bull Soc Path exot* 43 151-156 1950

63 Taylor D J Greenberg, J, Highman, B and Coatney, G R Experimental infection of guinea pigs with *Endamoeba histolytica* *Am J Trop Med* 30 817-828 1950

64 Reyniers J A Germ-free life studies LOBUND Reports, Univ of Notre Dame 1 1-120, 1946

65 DALE, H H, and DOBELL, C Experiments on the therapeutics of amoebic dysentery J Pharmacol & Exper Therap, 10 399 439, 1917
66 CLAMPIT, J M Comparative therapy of Endamoeba histolytica infections in the kitten Am J Trop Med, 28 289-294, 1948
67 THOMPSON, P E and LILLIGREN, B L Chemotherapy of experimental Endamoeba histolytica infection in dogs Am J Trop Med, 29 323-338, 1949
68 FAUST, E C, SCOTT, L C, and SWARTZWELDER, J C Influence of certain foodstuffs on lesions of Endamoeba histolytica infection Proc Soc Exper Biol & Med, 32 540-542, 1934
69 ANDERSON, H H, JOHNSTONE, H G, and HANSEN, E L Experimental chemotherapy of amebiasis Am J Trop Med, 27 153-160, 1947
70 ANDERSON, H H, HANSEN, E L, SAH, P P T and CAFISO, J R Amebacidal and pharmacologic activities of carbarsone oxide (P-carbamidophenyl arsenous oxide) and its dithiocarboxymethyl and dithiocarboxyphenyl derivatives J Pharm & Exper Therap, 91 112-123, 1947
71 ANDERSON, H H, JOHNSTONE, H G, BOSTICK W, CHEVABRIA, A P and PACKER, H Thioarsenites in amebiasis J A M A, 140 1251-1258, 1949
72 ANDERSON, H H and ANDERSON, J VAN D Antibiotics against amebiasis in macaques Am J Trop Med, 30 193-201, 1950
73 HRENOFF A K Treatment of monkey amebiasis with terramycin J Parasitol, 37 329-330, 1951
74 ANDERSON, H H HRENOFF A K, ANDERSON, J VAN D, NAKAMURA, M and CONTOPOULOS A N Fumagillin in amebiasis Am J Trop Med & Hyg, 1 552-558, 1952
75 LEAKE, C D Chemotherapy of amebiasis J A M A, 98 195-199, 1932
76 TREVAN, J W Discussion at the January meeting British Pharmacological Society, 1947 (Unpublished)
77 BRINDLEY, W H and PYMAN, F L The alkaloids of Ipecacuanha Part IV J Chem Soc, 1067, 1927
78 GOODSON, J A, GOODWIN, L G, GORVIN, J H GOSS, M D, KIRBY, K S, LOCK, J A, NEAL, R A SHARP, T M and SOLOMON W The chemotherapy of amoebiasis Part II Amines derived formally from emetine Brit J Pharm & Chemotherap, 3 49-61, 1948
79 GOODSON J A GOODWIN, L G, GORVIN, J H, GOSS M D KIRBY K S, LOCK, J A, NEAL R A, SHARP, T M, and SOLOMON W The chemotherapy of amoebiasis Part III Variants of bis (diamylamino) decane Brit J Pharm & Chemotherapy, 3 62-71, 1948
80 FULTON, J D JOYNER L P, KING H OSBOND J M and WRIGHT, J Amoebicidal action and chemical constitution Proc Roy Soc London S, B 137 339-366 1950
81 SCHÖNHÖFER F Chemotherapeutisch Wirksame organische Basen bei der Entamoeba histolytica Fiat Rev German Science 1939-1946 Chemotherapy 35-95
2 NEAL R A The duration and epidemiological significance of Entamoeba histolytica infections in rats Tr Roy Soc Trop Med & Hyg, 45 363 370, 1951

83 LUTTERMOSER, G W, HASKINS, W. T, COLEMAN, N and JUMPER, J. R.: Experimental *Endamoeba histolytica* infections in rabbits with reference to chemotherapy *Am J Trop Med & Hyg*, 1.162-170, 1952
84 TAYLOR, D J and GREENBERG, J Experimental chemotherapy of *Endamoeba histolytica* infections in the guinea pig *Am J. Hyg*, 56 58-70, 1952
85 McCOWEN, M C, CALLENDER, M E, and LAWLIS, J. F, JR. Fumagillin (H-3), a new antibiotic with amebicidal properties *Science*, 113 202-203, 1951
86 BANKS, C K Organometallic compounds as chemotherapeutic agents, in *Advancing Fronts in Chemistry Vol II. Chemotherapy*, New York, Reinhold Publishing Company, 1946, 156 pp
87 HASKINS, W T, LUTTERMOSER, G W., and BRADY, F. J: The physiological disposition of diodoquin, vioform, and chiniofon in the rabbit as determined with radioiodine *Am J Trop Med*, 30 599-612, 1950.
88 HASKINS, W T and LUTTERMOSER, G W.: Spectrophotometric determination of 8-quinolinol and some of its halogenated derivatives. *Analytical Chem*, 23 456-458, 1951
89 PALM, A Untersuchungen in der Chinolinreihe, X Mitteilung: Dijodverbindungen *Arch Exper Path. u Pharm*, 166 176-185, 1932
90 PARMER, L G On the relative efficacy of emetine in intestinal and hepatic amoebiasis *Proc Soc Exper Biol & Med*, 68 362-363, 1948
91 ANDERSON, H H and LEAKE, C D The oral toxicity of emetine hydrochloride and certain related compound in rabbits and cats *Am J Trop Med*, 10 249-259, 1930
92 ALBRIGHT, E C, TABERN, D L and GORDON, E S. The metabolism of chiniofon using radioactive iodine *Am J Trop Med*, 27.553-560, 1947.
93 KNIGHT, A A and MILLER, J Comparative studies on the iodine absorption of anayodin, chiniofon, diodoquin, and vioform in man *Ann Int Med*, 30 1180-1187, 1949
94 REES, C W TAYLOR, D J and REARDON, L. V: The presence of *Endamoeba histolytica* in the liver of guinea pigs with experimental intestinal amebiasis *J Parasitol* In press
95 REINERTSON, J W and THOMPSON, P E: Experimental amebic hepatitis in hamsters *Proc Soc Exper Biol & Med*, 76 518-521, 1951.
96 THOMPSON P E and REINERTSON, J W Chemotherapy of amebic hepatitis in hamsters with emetine, chloroquine, amodiaquin (Camoquin), quinacrine and other drugs *Am J Trop Med*, 31 707-717, 1951

CHAPTER 7

CLINICAL NOTES: ON THE ETIOLOGY, PATHOLOGY, CLINICAL MANAGEMENT AND THERAPY OF AMOEBIASIS

By

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Clinical Notes†

Definition

Amoebiasis is the appropriate term for intestinal infections due to *Entamoeba histolytica* "Amoebic dysentery," although commonly used loosely in some circles, should be restricted to those cases actually exhibiting dysenteric stools In the U S A, these are uncommon

Pathogenesis in man

The biological cycle of *E histolytica* comprises two forms of the organism, the trophozoite and the cyst Trophozoites are the active vegetative forms which accomplish tissue invasion These encyst under appropriate conditions, and, when mature have four nuclei It is in this form that the amoeba commonly infects man orally, the cyst wall protecting the organism from gastric juice Excystation occurs in the upper bowel, liberating four small trophozoites, each of which divides immediately, thus producing eight young trophozoites from a single cyst So far as is presently

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†AUTHOR'S NOTE In these last pages, pertinent clinical aspects are presented in a considerably condensed form The development of a pattern of clinical thinking in this chapter renders some repetition of material included in earlier pages unavoidable Further in common with most synopses it has not been possible to avoid entirely a dogmatic ring on some pages This seems to be an inexorable price of brevity

known, encystation within the host takes place in the lumen of the bowel. Encystation has never been satisfactorily demonstrated actually within the tissues. In general, trophozoites are found in diarrheic or dysenteric stools, and the cysts are expected in formed stools. The fundamental factors in actual tissue invasion are far from clear at this time. Some are operative through influences on the host, others through the parasite. While it is usually difficult to determine the relative importance of given factors in an individual patient, it is well to consider this aspect at large.

In relatively recent years, more attention has been paid in this country to the existence of two "races" of *E. histolytica*. The principal distinguishing feature between them is size: large race, cysts greater than 10 to 11 micra in diameter; small race, cysts less than 10 to 11 micra in diameter. The trophozoites are larger than cysts, but the corresponding differences remain. Admittedly cyst diameter is far from an ideal criterion, but, in practice, it does appear that the small race is far less pathogenic for man than the large variety, and, thus, should be identified if possible. It appears that many of the amoebic infections occurring in the U.S.A. are due to the small race. It will be some time before adequate epidemiologic data are collected on this point because the "race" of *E. histolytica* is not yet routinely designated by all laboratories making the reports. Then, even among the so-called "large race" amoebae,* there may be strain differences in invasiveness and virulence. These differences are even more difficult to assess in clinical practice but are demonstrable in animal inoculation studies in the laboratory.

The enteric bacterial associates exert very important influences on pathogenicity. Laboratory studies have produced many data illustrating the effects of varying the cultural associates of the amoebae on the pathogenicity of the parasite in animals. Recent studies utilizing germ-free animals demonstrated that even in these highly vulnerable subjects, *E. histolytica* may possess the ability to produce disease only when suitable bacterial associates

*"Amoebae" here and elsewhere refers to *E. histolytica*.

are present These data are largely preliminary at present and final conclusions would be premature at this time In clinical studies considerable attention has been given to identifying these floral requirements specifically but have not been completely successful It may be that the overall nature of the enteric flora is more important than the presence of any particular bacterial species Epidemiological and clinical studies have pointed out that in some areas an increased incidence of diarrhea or dysentery due to amoebiasis is related to poorly balanced diets—especially low-protein diets Parallel investigations of populations with good nutritional status disclosed a significantly lower incidence of *symptomatic* amoebic infections, although the overall prevalence of infection was as high or higher than the poorly-nourished community with much symptomatic amoebiasis

At this time, it appears that, with other factors remaining equal, the influence of the general diet, mediated through the changes thus induced in enteric flora, is very important in determining the pathogenicity of *E histolytica* Other factors which doubtlessly play roles in determining the susceptibility to amoebiasis and the degree of pathology developed are host resistance and relative immunity, pre-existing trauma to colonic mucosa, and fecal stasis Amoebic ulcerative lesions are more common in the regions of the colon in which stasis of the fecal stream occurs

Pathology Intestinal

The most severe lesions are seen in patients exhibiting clinical symptoms, but a surprising degree of gross pathology may develop with minimal symptomatology The initial mucosal lesion is a small, superficial, pin-point crater exhibiting a yellowish center and erythematous areola, surrounding mucosa is normal unless the process is very far advanced or complicated by other forms of colitis As local invasion progresses, the ulcer widens into a flask-shaped lesion when the process reaches the relatively nonresistant submucosa Ordinarily the ulcer extends down to the muscularis mucosa layer and spreads laterally Undermining occurs and the over-hanging edges of the ulcer build up Perforation can occur but is infrequent The uncomplicated lesion presents a ... specific

Eighty proved cases have been found in the literature. The lesions are usually situated in the cerebral cortex (carotid artery distribution) and consist essentially of tissue destruction with a delayed granulocytic response Secondary infection is common.

Cutaneous amoebiasis is found around fistulous openings of bowel or other abscesses, around the anus, or colostomy drainage sites Skin destruction may be extensive, the ulcer presenting a dull base, and over-hanging edges. The skin around the lesion characteristically presents a dusky cyanotic hue. All organs of the genitourinary tract have been involved, but these complications are rare In an extensive literature review, reports of amoebic lesions in practically every organ may be found. Though some of these may be doubted, it would still appear that no tissue is completely resistant to this organism

Clinical Manifestations

Chronic intestinal amoebiasis

This is the most common form of amoebiasis seen in this country The infection may be·

a) asymptomatic,

b) manifested by vague abdominal discomfort, poor appetite and digestion, malaise and other non-specific systemic complaints, or

c) manifested by more definite colonic complaints such as diarrhea, dysentery, constipation, pain, etc.

(a) Essentially asymptomatic infections apparently occur far more commonly than clinically apparent ones Many of these are associated with the small race *E. histolytica*, but the large race infections may also be silent. Careful clinical and laboratory examinations do not show any evidence of pathology in many patients in this category In some, however, rectosigmoidal ulcers are demonstrable though there are no complaints Therapy is indicated largely to prevent progression of the process and possible complications and for public health reasons.

(b) In this category, symptoms are often so vague and protean in nature that their amoebic etiology is justifiably controversial,

even though they may improve or disappear after anti-amoeb
therapy However, in many of these patients, colonic mucos
lesions are present, and to an advanced degree, out of keepin
with the mildness of their complaints. In those symptomatic pa-
tients in whom the proctosigmoidoscopic examination is negative,
it should be recalled that amoebic lesions predominate in areas far
beyond the limit of this instrument Barium enema studies may
show cecal deformities, or other abnormalities, but even this ex-
amination may be negative in the presence of significant lesions
On physical examination, cecal tenderness is practically universal.
Tenderness over other parts of the colon is variable In some
patients, a non-tender or slightly tender hepatomegaly is found
This differs from classical amoebic hepatitis and resolves after
adequate therapy of the intestinal infection, without resorting to
drugs usually required for extra-intestinal lesions (v i) Various
systemic findings may be found depending on the degree of
gastrointestinal disturbance.

(c) Patients in this cateory present symptoms which rather
sharply focus attention to the large bowel, and in these, amoe-
biasis is least often missed Abdominal aches and cramps, flat-
ulence, recurrent diarrhea or dysentery, qualitative or quantitative
dyspepsia, anorexia, chronic fatigue and weight loss are common
complaints Sometimes alternating diarrhea and constipation oc-
cur, but this is unusual Headaches, arthralgias and other non-
specific symptoms are seen As might be expected, patients in
this group are most vulnerable to the complications of amoebiasis

A wide range of pathology is demonstrable by proctosigmoid-
oscopy Multiple small ulcers, or advanced coalesced and second-
arily infected lesions may be found Considerable damage is in-
curred in long-standing disease Proctosigmoidoscopy and barium
enema studies are essential to a complete appraisal of these pa-
tients Abdominal palpation discloses colonic tenderness, particu-
larly over the lower quadrants. Inflammatory masses may be
detected and the hepatomegaly described above is commonly en-
countered The salient features of other clinical forms of amoe-
biasis and features of management are briefly discussed (v i)

Acute intestinal amoebiasis (dysentery)

The infection may develop in fulminating proportions with fever, chills, intense abdominal cramps, tenesmus and bloody stools The severely dysenteric stool contains conside amounts of cellular debris, pus (less than in bacillary dysent blood, Charcot-Leyden crystals and *E histolytica* trophozo In general, the frequency of stools is usually less than that s in bacillary dysentery and they contain more fecal mate Physical findings are in direct ratio to the severity of the ac process Proctosigmoidoscopy is of considerable value. Witho curative therapy, the acute episode may be fatal, or spontaneo remission with subsequent relapses may occur, with the infectio finally becoming chronic.

Intestinal complications

Acute amoebic appendicitis occurs in acute or chronic intestinal amoebiasis On clinical examination it is indistinguishable from non-amoebic appendicitis, and definitive diagnosis is often made histopathologically. Preoperative diagnosis of the amoebic etiology is of great importance, because, without anti-amoebic medical therapy, the postoperative course is usually stormy Adequate stool examinations should always be made when there is the smallest suspicion of amoebiasis in a patient presenting clinical signs of appendicitis. Amoeboma is to be differentiated from carcinoma Medical therapy is always indicated, though surgical excision is sometimes necessary when destruction is far advanced or obstruction is complete. Hemorrhage of a significant degree is rare Replacement of blood loss and anti-amoebic therapy are required Intestinal perforation is usually seen only in severe acute cases Due to prior fibrosis, the subsequent peritonitis is usually localized, but generalized peritonitis has been reported.

Extra-intestinal complications

Amoebic hepatitis occurs predominantly in males. The principal clinical features are painful hepatomegaly, cough, fever, and weight loss There is often no history suggestive of precedent

intestinal amoebiasis. Evidence of a pneumonitic process in the right lower lung field is a common finding. Chest roentgenograms disclose elevation, but usually not fixation, of the right diaphragmatic leaf. Salient laboratory features are moderate leucocytosis with a normal differential count, positive complement-fixation test, mildly impaired or normal liver function tests. The stools may be negative for *E. histolytica*. The response to specific therapy (*v.i.*) and post-treatment decline in complement-fixing antibodies are important diagnostic features.

Amoebic liver abscess may be acute or chronic. In the acute phase the clinical picture resembles that described above for amoebic hepatitis, but is more advanced. Fever tends to be of the septic type, leucocytosis is more marked, and diaphragmatic elevation tends to be fixed. When abscesses are multiple, mortality is considerably increased. The abscess may reach a subacute or chronic phase before the patient seeks treatment. A comprehensive review of amoebic liver disease is beyond the scope of this chapter, and the reader is referred elsewhere for a complete clinical review. It is important to note, however, that *the classical picture may be greatly altered if the abscess is located in the left or the caudate lobe and under these circumstances laparotomy may be necessary for diagnosis*. The material obtained by aspiration of the abscess cavity is characteristically reddish-brown and thick. Amoebae are not usually present in this "pus," as they reside in the walls of the lesion. As with pre-suppurative hepatic amoebiasis, response to specific therapy and aspiration and serial post-treatment complement-fixation reactions aid in diagnosis.

Laboratory Diagnosis

Inasmuch as technical aspects are covered in an earlier chapter, little remains to be said here. A few comments are presented as guides to a laboratory work-up when the infection is suspected. The stool examination "battery" should consist, at least, of a direct preparation and a concentration technique. If practicable, an iron-hematoxylin stained preparation and culture may be added. There is no hard-and-fast rule governing the number of stools to be so examined. In general, we have preferred to ex-

amine at least five specimens passed over a 12 to 14 day period before ruling out the infection When amoebiasis is strongly suspected, this series is extended When purged specimens are preferred, the first post-purgation passage is discarded and subsequent ones submitted to laboratory examination. Material obtained at proctoscopy should be immediately examined. Great caution should be exercised with these specimens, with meticulous attention to morphologic criteria, as many "false-positive" identifications are made by the unwary on macrophages and epithelial cells In the well-formed stool, *E. histolytica* cysts are more likely to be present than trophozoites Both forms of the parasite are found in soft or diarrheic stools In the severe diarrheic or dysenteric stool, trophozoites predominate

The complement-fixation test, at present, is most valuable in the diagnosis and follow-up of extra-intestinal amoebiasis Its service in uncomplicated intestinal amoebiasis is limited. Methods and results vary in different laboratories, and it is important to know the limitations of the procedure in the laboratory serving you.

Anti-amoebic Therapy

Intestinal infections

On the basis of present knowledge, all infections with *E. histolytica* should be treated The therapy should probably encompass the intestinal flora as well as the parasite itself. Since silent metastatic amoebic lesions may co-exist in the liver, it appears rational to choose therapy which will also attack these sites. This is particularly important in those patients with significant colonic lesions The ideal chemotherapeutic agent against amoebiasis has not yet been recognized. This field remains dynamic and controversial, and a comprehensive review is not feasible here. However, a few general remarks on the comparative efficacies of current agents can be offered

The older amoebicides, the iodohydroxy-quinolines, carbarsone, etc. are generally less efficient than the wide-spectrum antibiotics in symptomatic patients *Oxytetracycline* (Terramycin) is highly effective against intestinal lesions and appears superior to Chlortetracycline (Aureomycin). Neither antibiotic is trustworthy

against hepatic amoebiasis. *Fumagillin*, although highly amoebicidal *in vitro*, fails in a significant number of acute cases. It is not effective against extra-intestinal lesions, and its toxicity has not been fully evaluated. *Milibis* appears to be less efficient than the wide-spectrum antibiotics. Early experience with *Erythromycin* has been favorable. There is evidence that this agent is active in intestinal and extra-intestinal sites. The role of chloroquine and emetine in intestinal amoebiasis is discussed below.

Extra-intestinal infections

For pre-suppurative lesions (amoebic hepatitis, pneumonitis, skin lesions, etc), *chloroquine diphosphate* is the first drug of choice. It is usually well tolerated and effective in doses (adult) of 1.0 gm for the first two days and 0.5 gm for the next 12 days. This dosage of chloroquine is also used as adjunctive therapy, with an antibiotic, for symptomatic bowel infections when silent metastatic foci may exist (*v.s.*). Prior to the advent of chloroquine therapy, emetine hydrochloride was the only drug available for extra-intestinal amoebiasis. Its inherent toxicity limits its use, and the therapeutic margin of safety is narrow. Since chloroquine possesses equal effectiveness, the present indications for emetine are restricted. It is of value in chloroquine-refractory cases. The *maximal* dose for a *well-developed adult male* is 60 mg./day for no more than 10 days, by subcutaneous route. For severe dysentery, emetine HCl is used as symptomatic therapy to relieve the intense cramps and tenesmus. The basic daily dose is 60 mg./day, but it should be continued only until these symptoms are controlled. It is never given for more than 10 consecutive days and often a shorter course is sufficient. Used for this indication, it is not expected to clear the intestine of the parasite.

Localized amoebic abscess is treated with closed drainage (aspiration) plus chloroquine or emetine plus antibiotic therapy to avoid secondary infection. Under some circumstances, enzymatic debridement of the abscess cavity is indicated to facilitate evacuation. Abscesses located in the left or the caudate lobe often require drainage at laparotomy due to the inherent danger of "blind" puncture of these sites.

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This Book

PROBLEMS IN AMOEBIASIS

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